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THE EFFECT OF RELATIVE HUMIDITY ON AIRBORNE BACTERIA

By

J. M. DALLAVALLE, T. W. KETHLEY,
W. B. COWN and E. L. FINCHER

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SUMMARY

During the period 1 January to 31 December 1957 a variety of approaches were employed to investigate the role of water in determining the fate of airborne bacteria. Rates of dehydration and rehydration of microscopic particles of a proteinaceous material were measured. Phase contrast reversal was adapted to the measurement of the water content of living cells employed in aerial experimentation. Bacterial cells much larger than previously employed in aerobiological studies were successfully cultured. Results of various studies indicate: presence of a possible toxic factor in stationary cultures of bacteria; correlation between aerial death rate and length of initial stationary phase of growth of collected airborne bacteria; surface dehydration of agar is not the cause of conflicting results in using various samplers; washed cells can act as condensation nuclei in the air; and water content of living airborne bacteria is much greater than that of nonliving cells. More exact techniques are under development for determining the effect of high concentrations of materials on bacterial survival and effects of sudden changes in relative humidity on airborne bacteria.

PREFACE

This progress report covers the activities carried out during the period 1 January to 31 December 1957, the first year of a 2-year study. Almost all of the research herein described is the outgrowth of earlier studies which were reported previously. Because of the specialized nature of the equipment developed to carry out many of these investigations, the appropriate sections of the final report (Project No. B-106) of the earlier investigations, which describe this equipment in detail, have been reprinted as an Appendix to this progress report.

The results of the researches herein reported have been grouped into separate and discrete sections; these represent a series of more or less parallel investigations which were planned to lead toward the final objective--a better understanding of the role of water in determining the fate of airborne microorganisms. Separate, parallel studies were outlined in order to better utilize the special facilities and particular qualifications of the personnel devoted to this work. The original estimate of the scope of the research indicated that the current year of effort (that is, the second for the total project) would be adequate to interrelate these separate studies, to plan and execute critical experiments indicated by the results of these studies, and to reach some plateau of understanding on the road to ultimate comprehension of the role of water in determining the fate of airborne microorganisms. Certain studies have revealed unexpected results, however, throwing more emphasis on problems not previously considered of major importance, and some studies have produced clues to problems not originally envisioned. In addition, developmental studies involved in new techniques necessary for the solution of some

of the separate problems have delayed the scheduled program of action. For these reasons, this progress report has been prepared to present the accomplishments of the first year, to indicate the manner in which these will be utilized during the current year, and to show the need for at least another year of effort on the primary research objectives.

I.

RATES OF DEHYDRATION AND REHYDRATION OF PARTICLES OF BEEF EXTRACT

The rate of dehydration of atomized droplets containing bacteria has, at various times, been considered a determining factor in the ultimate fate of airborne bacteria. In our own work, we have maintained that the time involved in holding the droplets in the prechamber following atomization (about 30 minutes) is more than sufficient to allow for complete equilibration of the droplets. Likewise the time involved in passing particles from the prechamber to the chamber or cylinder (about one minute) is more than sufficient for equilibration of the particles. That is to say, we have felt justified in considering the particles in the chamber or cylinders as being in a state of equilibrium with the conditions existing there. The rate of dehydration, therefore, should play no significant role in determining the fate of the airborne bacteria under study in the chamber or cylinders, although it might be important in determining the number of survivors which actually enter the chamber or cylinders. The results of our previous studies on the effect of a sudden change in relative humidity on airborne bacteria have indicated the above assumptions to be correct.

As a rough estimate, values were assigned to the time required for dehydration of the experimental bacterial aerosols in terms of evaporation of water. It was concluded that the rate of equilibration would be dependent upon the relative humidity[1]. In order to obtain more exact values of rates of evaporation, measurements were made of the time required for droplets of solutions of

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[1] T. W. Kethley, W. B. Cown, and E. L. Fincher, "The Nature and Composition of Experimental Bacterial Aerosols," Applied Microbiology 5, 1-8 (1957).

beef extract (as representative of proteinaceous materials associated with bacterial aerosols) to dehydrate. Measurements were also made of the time required for the dried particles to rehydrate. To determine the rate of evaporation of water from the initial droplet, solutions were atomized directly upon a polyethylene film mounted upon a microscope stage. The actual atomizer nozzle was only a centimeter or so distant from the polyethylene film. Under these conditions it was assumed that the droplets observed were of essentially the same diameter as produced at the atomizer tip, because of the close proximity of the observational field and the atomizer tip, and because, since the droplet maintains its spherical shape, the polyethylene film is not wetted.

It was not possible to determine the rate of water loss of droplets having diameters less than 100 microns because of the necessity of observing and estimating the initial diameters at the exact instant the droplets struck the polyethylene film. Measurements on these droplets in atmospheres of known humidity showed that the transition from droplet to particle progressed as predicted, that is, in exactly the same time as equal spheres of water under the same conditions. We have considered this previously (see Footnote No. 1) in terms of Houghton's[2] treatment of the evaporation of water from spheres, and concluded that even at 99-per-cent relative humidity atomized droplets of the size employed in our studies would equilibrate in a few seconds.

Because of the small changes in size of particle involved in dehydration, the exact determination of the time required for dehydration of the particles

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[2] H. G. Houghton, "A Study of the Evaporation of Small Water Drops," Physics 4, 419-24 (1933).

resulting from the evaporation of water from the droplets at 99-per-cent relative humidity required more elaborate equipment than that used to estimate the rate of evaporation from the initial droplet. The equilibrium moisture content of beef extract at 95-per-cent relative humidity was found to be 75-per-cent water. Below 20-per-cent relative humidity the equilibrium moisture content is essentially constant at 10-per-cent water. Thus, an initial droplet of 0.3-per-cent beef extract broth of 13 microns diameter (the average size produced in our routine experimental work) would evaporate to a particle of 2.8 microns diameter at 95-per-cent relative humidity; this particle would dehydrate to a diameter of 1.8 microns at humidities below 20 per cent.

In order to measure accurately the small changes in particle diameter involved in the dehydration of particles of beef extract broth, the equipment developed by the Micromeritics Group for the study of dehydration of NaCl particles was employed. A diagram of this equipment is shown in Figure I. 1. Particles suspended on fibers in the field of the microscope are equilibrated to either very high humidity and then suddenly exposed to very low humidity, or the reverse, in order to study either dehydration or rehydration of the particles. In either instance the camera operates at constant speed and by means of a calibrated grid, lapsed time and particle size can be measured. The particles are suspended on strands of methacrylate resin of less than one micron diameter. These strands are prepared by pulling out a viscous mixture of methacrylate resin and aniline oil until strands of the desired diameter are obtained. These are strung across a small glass chamber, and the particles of beef extract collected on the strands by atomizing a solution above

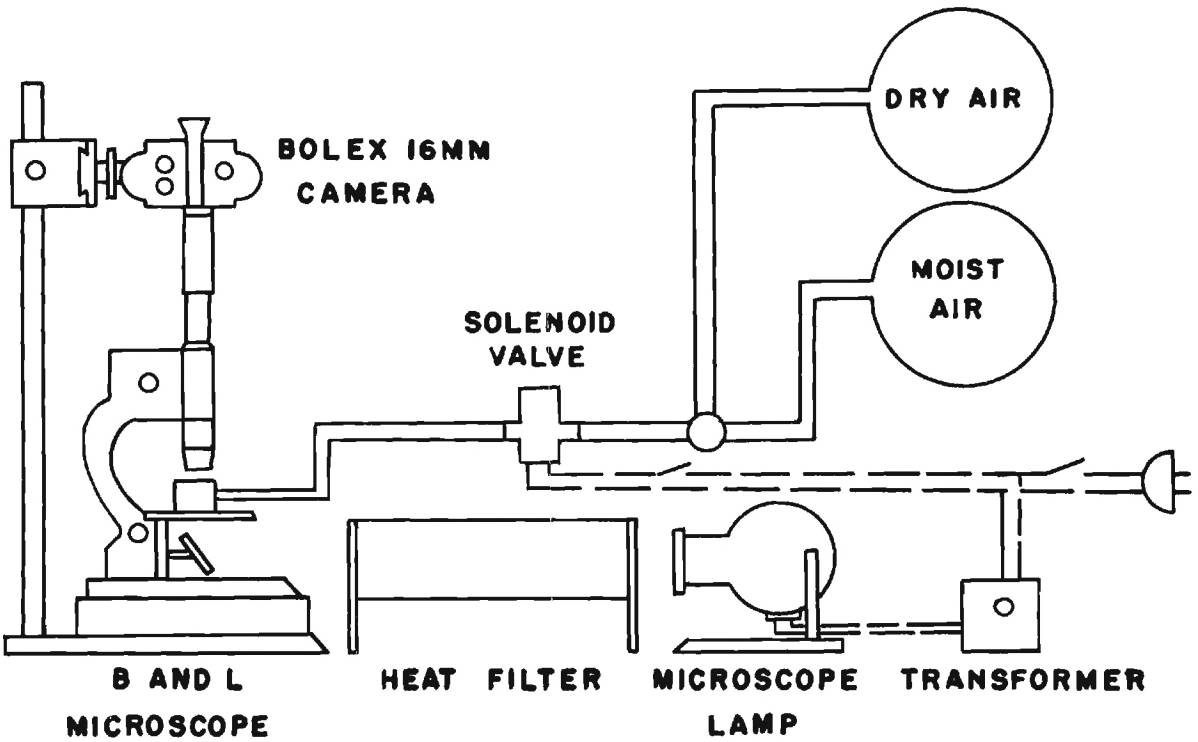

Figure I. 1. Schematic Diagram of Dynamic Hydrating Apparatus Arranged for Photographic Recording.

the strands. The chamber is placed on the microscope stage and connected to the dry and wet air sources.

Limitations in the depth of focus of the available optics made it impossible to measure with accuracy particles of diameters much less than 10 microns. For this reason 5-per-cent solutions of beef extract broth were atomized and particles collected for examination, rather than the more dilute solution employed in previous work. Particles of 8 to 20 microns diameter were obtained and measurements made on the rate of dehydration and rehydration in the apparatus described above. Representative data are shown in Figure I. 2. Because of the problem of maintaining an exactly uniform temperature throughout the system, a precise value for the relative humidity of the moist air is not given; reference is made only to transition from high humidity to low humidity, or the reverse. Computations based on solids content of the particles indicated that the average relative humidity never exceeded 95 per cent. This is probably due to heating effects from the microscope lamp, although these were minimized as much as possible by a heat filter, and by turning the lamp on for alternate frames, while running the camera continuously. An increase of 1° F in the internal temperature of the system would account for a shift from 99-per cent to 95-per-cent relative humidity.

Although the data obtained on the dehydration and rehydration of particles of beef extract have not been completely analyzed, initial treatment has revealed some factors of considerable interest. Close examination of the information shown in Figure I. 2 shows that particles of approximately the same size when dry do not necessarily rehydrate to the original wet particle diameter.

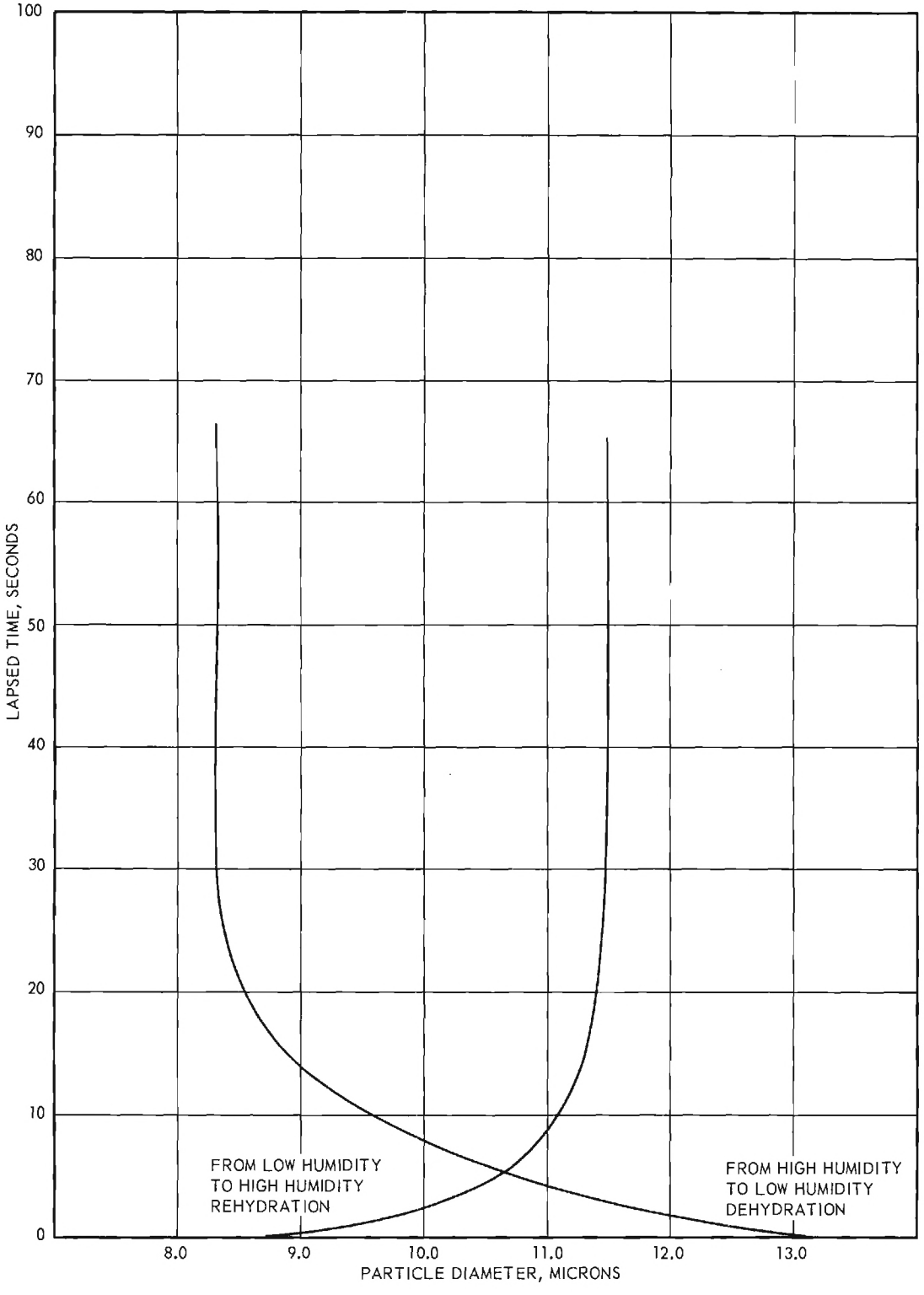

Figure I. 2. Representative Data for Dehydration and Rehydration of Particles of Beef Extract.

The values shown for the rehydration of a particle in this figure are for a particle which had been dehydrated and then rehydrated; it has not rehydrated completely. This response appears to be characteristic; the more often a particle is cycled from wet to dry and back again, the less well it rehydrates. This type of response might be a factor in the survival of dust-borne bacteria, and is being explored further through more quantitative measurements of rehydration in atmospheres of known humidity.

The two curves in Figure I. 2 also illustrate the finding that, for particles of equal diameter, rehydration times are essentially the same as dehydration times. This is an unexpected finding, because the driving force promoting dehydration is primarily the difference between the vapor pressure of the aqueous complex making up the particle, and the vapor pressure of water in the ambient air, while rehydration involves condensation and solution as well. This suggests that internal diffusion may be the determining factor in dehydration. If so, the rate of dehydration would be a function of particle size, and in order to verify this the range of particle sizes was increased. Measurements were made in the 0.1-micron diameter range with equipment developed by the Micromeritics Group. In this equipment, as shown in Figure I.3, very fine particles under high pressure (saturation humidity) are forced out of the expansion nozzle into the field of a light-scattering device. The stream of particles issuing from the expansion nozzle is strongly light scattering; as the particles dehydrate there is a change in scattering. Knowing the velocity of the stream, distance from the nozzle can be calibrated in milliseconds. By plotting distance from the nozzle against light scattering,

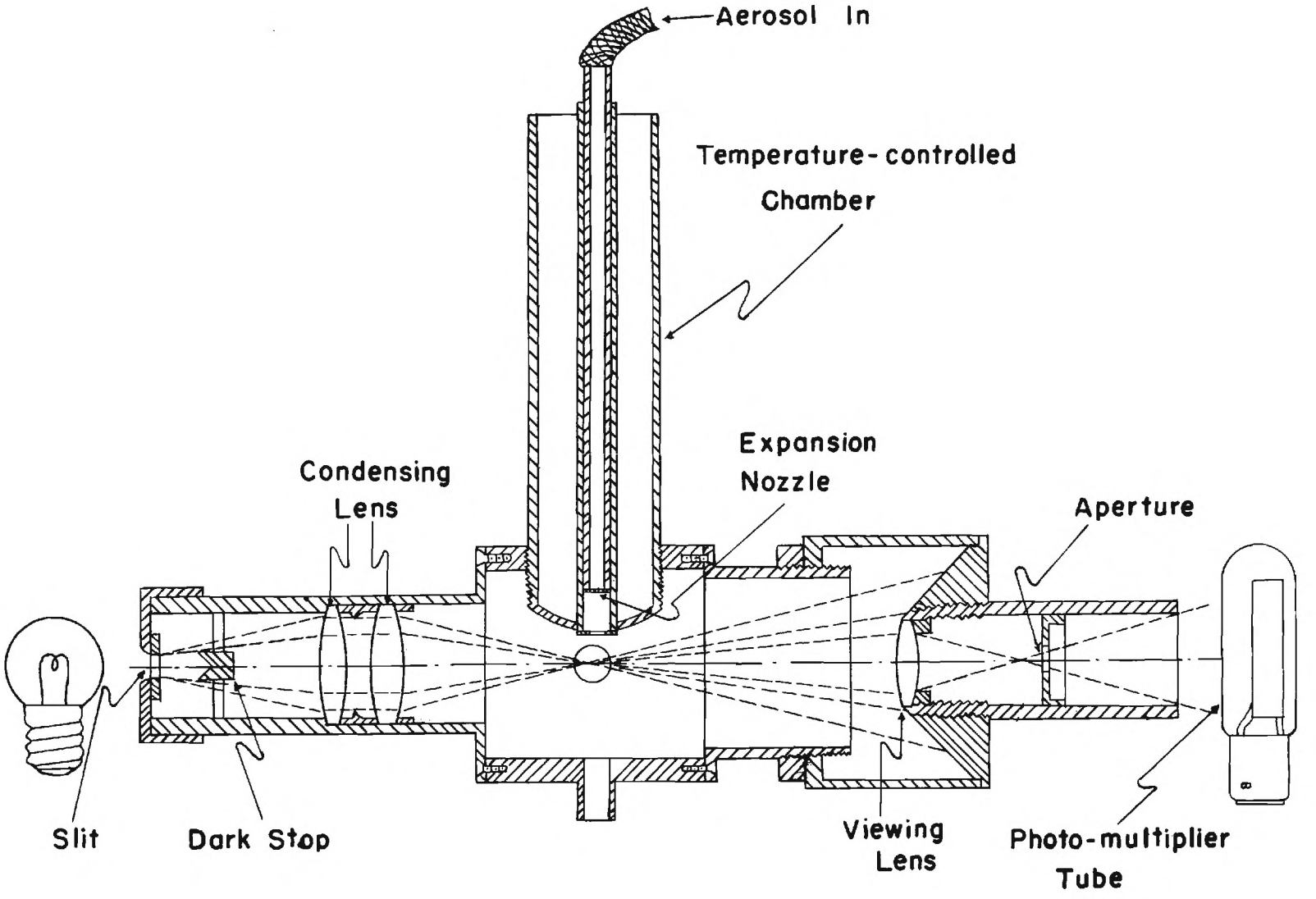

Figure I. 3. Schematic Diagram of Light-Scattering Device for Measurement of Rate of Dehydration of Small Particles.

the time required for dehydration can be determined. For beef extract particles of 0.08 microns diameter this time is 0.58 milliseconds.

The dehydration data for the several sizes of particles were assembled and the rates of moisture loss calculated in terms of diffusional drying, using Newman's graphical method.[3] Newman's method employs a theoretically derived plot of values for second-stage drying (that is, the stage following the evaporation of free water), in terms of the history of the solid during the drying process. This method assumes that a given material has a characteristic diffusion constant. This infers that the rate of diffusion of moisture through the solid is constant, the time required for drying depending upon the size of the solid and the final concentration of moisture in the solid. With particles of beef extract the final concentration of moisture will be determined by the relative humidity of the ambient air; this is the equilibrium moisture content of the material.

For graphical presentation, Newman's data are plotted using as the Y-ordinates a term which expresses the fraction of total moisture lost during the particular stage of drying. For the particles under investigation, the fraction of total moisture converts to fraction of volume change expressed in terms of diameters:

$$Y = \frac{d^3 - d_e^3}{d_o^3 - d_e^3}$$

where d is the final diameter, d_e the diameter at equilibrium moisture content,

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[3] A. B. Newman, "The Drying of Porous Solids: Diffusion Calculations," Trans AIChE 27, 310-333 (1931).

and d_o the initial diameter. In Newman's graphical presentation X-ordinates are in terms of moisture diffusion, and the same terminology is applicable for the drying of particles:

$$X = \frac{Kt}{r^2}$$

where K is the diffusion constant, and r is the radius of the particle at the end of the time, t.

A plot of Newman's data is shown in Figure I. 4, with averages of experimental observations shown as seconds on the curve. The average value of K was found to be 0.44×10^{-8} cm^2/sec for the moisture in the beef extract particles.

A chart such as the Newman plot for drying is actually a history of the moisture content of a porous solid during drying, and detailed information as to the probable concentration of water in a particle at any moment can be derived. This analysis has not been completed, but the closeness of fit of experimental to theoretical data as shown in Figure I. 4 indicates the value of such an analysis. The results are expected to aid in interpreting data relevant to "critical" moisture contents and death of bacteria.

Although the experimental data fit the theoretical values for second-stage drying in terms of the history of the moisture content during this process, our immediate concern was to evaluate the total equilibration time for different sizes of particles of beef extract. Because the Newman chart depicts the history of the moisture content of the particle, at equilibrium the value of Y is zero ($d = d_o$). Accordingly, 99.5-per-cent equilibration (Y = 0.005) was used instead of 100-per-cent equilibration. The theoretical value

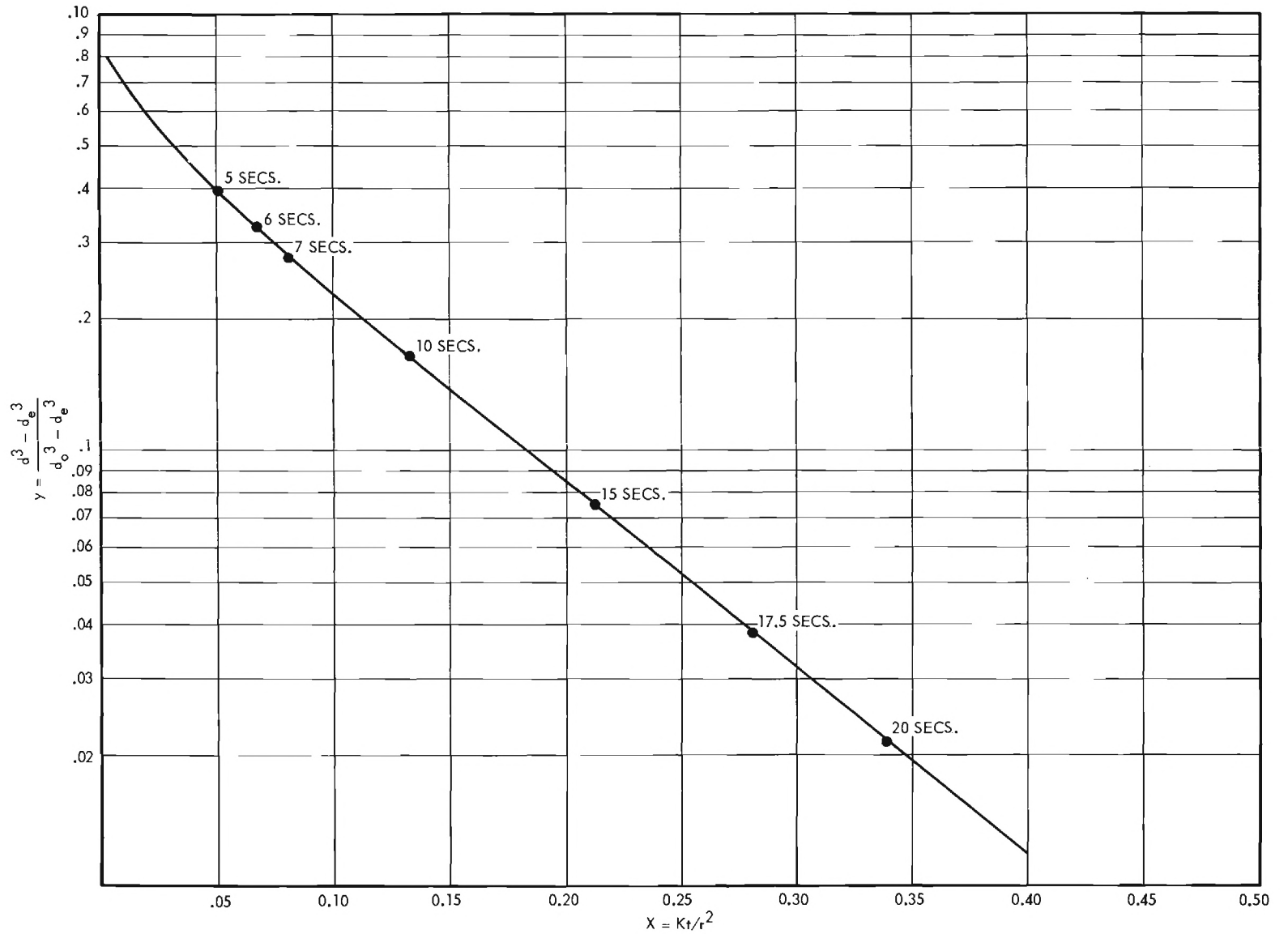

Figure I. 4. Experimental data for Dehydration of Particles of Beef Extract, Plotted on Newman's Theoretical Drying Curve.

of X for these conditions is 0.5. Converting the theoretical value of X into terms of microns and seconds and eliminating K, gives a statement for the dehydration time of a particle of beef extract. This statement is valid from approximately 95-per-cent relative humidity to less than 20-per-cent relative humidity:

$$t = (r^2)\ (1.14) \text{ or } t = (d^2)\ (0.285),$$

where t is the time in seconds for 99.5-per-cent equilibration, r is the radius and d the diameter of the dried particle. For the range of particle sizes studied (0.08 to 20 microns diameter) this equation fits very well, yielding values for t within ± 10 per cent of the observed values.

Simple computation of the relation between the solids content of beef extract at 95-per-cent and 20-per-cent relative humidities permits conversion of the value of the diameter of the dry particle into that for the diameter of the initial particle. For 95-per-cent and 20-per-cent relative humidities the ratio of diameters is 1.57; that is, multiplying the diameter of the dried particle by 1.57 gives the value for the initial diameter.

Similar treatment in terms of the fraction of initial moisture lost during the drying process yields the following equations for the 99.5-per-cent equilibration time for particles transferred from 95-per-cent relative humidity to some lower humidity, for example:

to 80-per-cent relative humidity; $t = (d^2)(0.052)$

$$d_o = (d)(1.29)$$

to 60-per-cent relative humidity; $t = (d^2)(0.085)$

$$d_o = (d)(1.38)$$

to 40-per-cent relative humidity; $t = (d^2)(0.195)$

$$d_o = (d)(1.50)$$

For the particles of beef broth employed in our experimental work (produced by atomizing 0.3-per-cent beef extract broth), which average 1.8 microns diameter at 20-per-cent relative humidity, the calculated drying time (from 95-per-cent relative humidity) is 0.85 seconds. This time is considerably less than that which we have allowed for final equilibration (one minute) of particles before entering the chamber or cylinders for study. The agreement between experimentally observed and theoretical values indicates that we are amply justified in our previous statements that we have been dealing with equilibrated particles in our chamber studies. In addition, the proof that the total time required for dehydration of a particle varies as the square of the particle diameter furnishes a basis for further studies on the effect of drying time on survival of airborne bacteria.

Although the above studies have been of value in determining dehydration times, and in indicating a method whereby moisture concentration at any time can be estimated in the dehydrating particle, the observations on rehydration have presented some new problems. In the first place, rehydration appears to proceed at approximately the same rate as does dehydration. No immediate explanation for this is available. In the second place, cycling the particles from wet to dry and back again caused the particles to rehydrate to moisture contents less than the original. This suggests that some change has occurred in the particle, making it less "wettable." Because of the potential value

of this observation in the study of dust-borne bacteria, these studies will be pursued further. A partial quantitation has been achieved through the use of a mathematical treatment similar to that of Crider, et al.,[4] and Milburn, et al.[5] The graphical analysis employed by these workers results from a plot of Y-ordinate values versus X-ordinate values:

$$Y = \frac{d}{d_o} \quad \text{and} \quad X = \frac{t}{d_o^2}$$

where d_o is the initial diameter of the particle, d the final, and t is the time for dehydration or rehydration for d_o to d. It is to be noted that X and Y ordinates are very similar to those employed by Newman. Newman's use of a Y-term describing the fraction volume change results in a straight line over the range of greatest interest, whereas the use of a Y-term describing the ratio of diameters results in an essentially nonlinear curve. The rehydration data could not be treated by Newman's method, however, and that of Crider, et al., and Milburn, et al. does show graphically the result of recycling, wet to dry and return, upon the rehydration of particles of beef extract. The measurements made on two such particles is shown in Figure I. 5. In this figure it is shown that the greatest value of d/d_o achieved by the particles during rehydration is 1.32 and 1.23. On the basis of original wet diameters, and supported by calculations based on the equilibrium moisture content of the

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[4] W. L. Crider, R. H. Milburn, and S. D. Morton, "The Evaporation and Rehydration of Aqueous Solutions," Journal of Meteorology 13, 540-47 (1956).

[5] R. H. Milburn, W. L. Crider, and S. D. Morton, "The Retention of Hygroscopic Dusts in the Human Lungs," A.M.A. Archives of Industrial Health 15, 59-62 (1957).

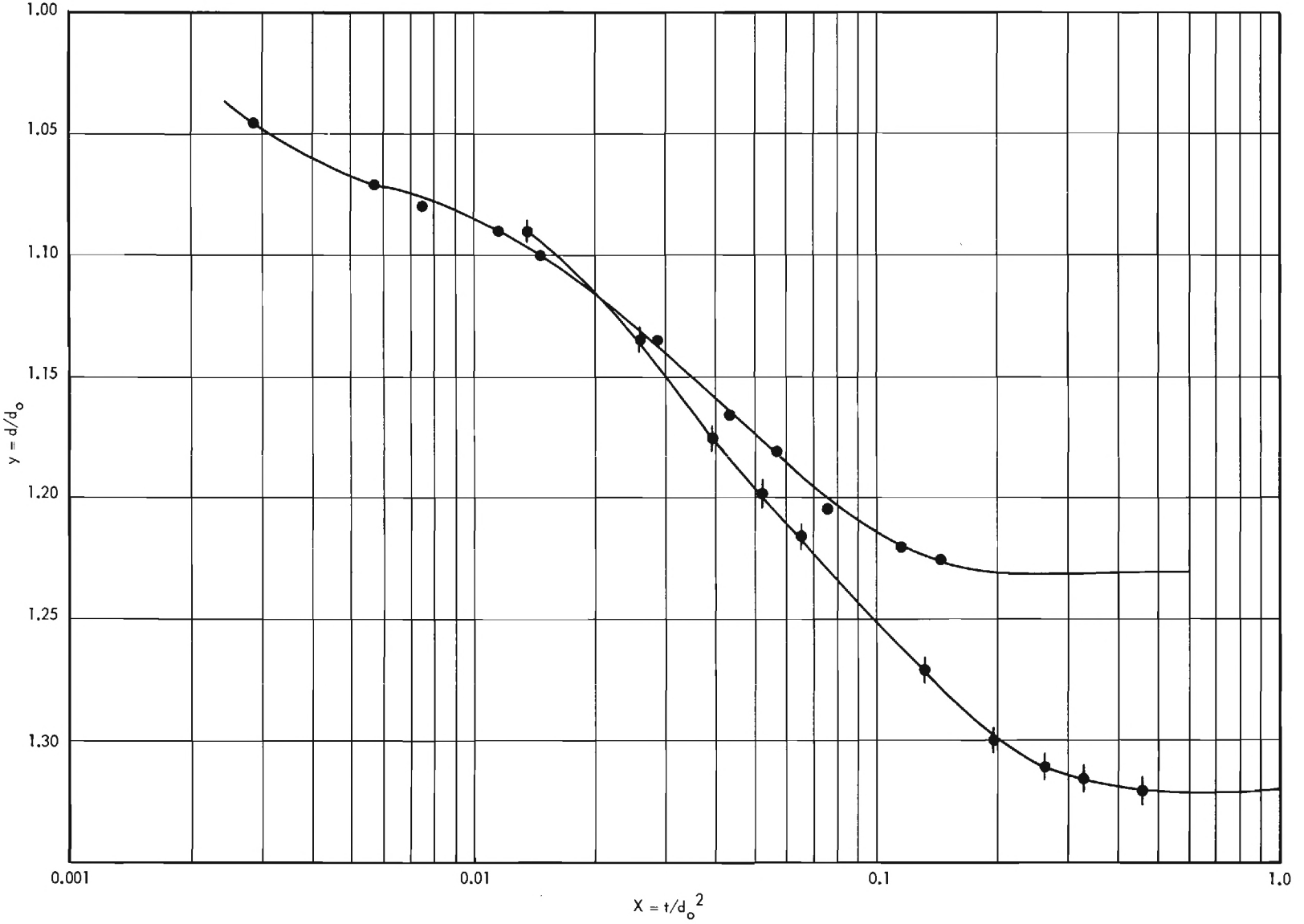

Figure I. 5. Rehydration Data for Particles of Beef Extract, Repeatedly Cycled from Wet to Dry.

beef extract, this value should be 1.57. The particle which rehydrated to 1.32 times its dry diameter was cycled once; that which rehydrated only to 1.23 times its dry diameter was cycled several times. However, some subsequent measurements indicate that the length of time a particle is held under conditions of low humidity is the important factor, rather than the cycling procedure. As indicated above, this phenomenon is being studied further, because of the possible significance in the interpretation of the condition of dust-borne bacteria, and also because of its significance in the rehydration of particles in the lungs.

Although the subject of dehydration and rehydration of airborne particles has been given a great deal of consideration, and we have established that our experimental bacterial aerosols in the chamber or cylinder are undoubtedly equilibrated with the conditions in the chamber or cylinder, we feel that the potential values of these studies have only been indicated. Further analysis is planned, particularly in estimating the moisture content of particles at any time during the process of equilibration in an attempt to determine the concentration gradients to which the bacteria in these particles are exposed during drying and to evaluate data relevant to a "critical" moisture content and the death of bacteria. In addition, investigations are planned on the effect of time of drying on the survival of airborne bacteria and on growth curves, based on the fact that time of drying varies with particle size. The results of these studies, in conjunction with the results of our previous studies on the effect of sudden changes in relative humidity on the survival of airborne bacteria, should yield an answer to the question as to whether or

not rate of drying influences the survival of airborne bacteria. Quantitative studies are already in progress to evaluate the factors which interfere with the rehydration of the particles of beef extract; it is hoped that the results of these investigations will be of value in the interpretation of studies on dust-borne bacteria and the rehydration of particles of proteinaceous materials in the lungs.

II.

ESTIMATION OF PROTEIN AND WATER CONTENT OF LIVING CELLS BY PHASE MICROSCOPY

One of the primary objectives of the current research of this group is to determine the water concentration of the living bacterial cell in the aerial state under various conditions. Recently, the results of several studies have been published by other workers, which indicate that vegetative cells gain or lose water freely, depending upon the relative humidity of the surrounding air.[1,2] However, the majority of the cells involved in the experiments reported have been nonviable. Adequate data concerning the water content of living vegetative cells under various conditions are not available. It should be noted that published studies dealing with the effect of sorbed water on the viability of bacterial cells[3,4] are not applicable to the problem of determining whether or not living vegetative cells gain or lose moisture when exposed to various humidities. Actually, these studies are based on equilibrium moisture data[5] for cells which were mostly nonviable.

The determination of the water content of living bacterial cells presents a number of problems. Proof that the cells under observation are alive is the most difficult problem, with a distinction between the water content of the

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[1] C. L. Stevens, "The Sorption of Water Vapor by Bacteria," Unpublished Manuscript. This paper gives values for the adsorption and desorption of water by lyophilized cells. Personal communication elicited the information that most of the cells were nonviable.

[2] D. G. Waldhalm and H. O. Halvorson, "Studies on the Relationship Between Equilibrium Vapor Pressure and Moisture Content of Bacterial Endospores," Applied Microbiology 2, 333-338 (1954).

[3] G. W. Monk, P. A. McCaffrey, and M. S. Davis, "Studies on the Mechanism of Sorbed Water Killing of Bacteria," Journal of Bacteriology 73, 661-665 (1957).

[4] G. W. Monk and P. A. McCaffrey, "The Effect of Sorbed Water on the Death Rate of Washed Serratia marcescens," Journal of Bacteriology 73, 85-88 (1957).

[5] Stevens, loc. cit.

substrate and that of the bacterial cell being next most difficult. These difficulties have thus far made it impossible to determine the water content of living bacterial cells in bulk. Data obtained in such studies indicates only that some cells are viable or nonviable, and that the mass under study has a certain water content. These data give no direct information concerning the specific water content of the particular cells which are viable. It must remain an assumption that there is a difference in response to external conditions between living and nonliving cells until direct proof is furnished that such a difference does or does not exist.

In the current researches an attempt was made to determine the water content of living bacterial cells by various indirect methods such as measuring the size of washed airborne cells at various humidities. However, the cells whose size is measured must be shown to be viable, and attempts to determine accurately both size and viability on particular cells free of substrate have thus far been unsuccessful. The one method which offers real promise in this study is that of estimating size of viables only from settling-plate data in the chamber. In this method, only viables are considered, size being estimated from the comparison of the concentration of viables in the air and the numbers of viables settling per unit time per unit area. As applied, the apparent rate of settling is a function of the sum of both mechanical and biological die-away; knowing one, the other can be determined. The mechanical die-away (the desired value in this instance) must be sufficiently large in comparison to the biological die-away to permit accurate determination. Attempts to devise a successful system have failed thus far. At present the problem is being attacked by searching for vegetative bacterial cells for which

either the biological die-away is extremely small or for which the mechanical die-away is extremely large. The former approach is being prosecuted by constant monitoring of air samples in a search for some bacterial species which is extremely well adapted to the aerial environment. One such organism has been isolated, but its cultural requirements have not been determined with sufficient exactness to warrant aerial studies (see under Sec. VIII). The approach to the estimation of the size of viable airborne bacterial cells involving an extremely large mechanical die-away has been prosecuted through a search for a bacterial species possessing a size greater than any of those which we have thus far examined. An exhaustive study of the literature indicated that Azotobacter agile might meet these requirements, and cultural studies leading to aerial testing are well advanced (see Section VII of this report).

The ideal method for determining the water content of a living bacterial cell would be to directly examine the cell, and then prove its viability by showing its ability to reproduce, grow, exhibit motility, or otherwise show signs of life. A most promising approach to this type of study is that of determining the index of refraction of the cell (assuming the protein concentration of the cell to be the chief factor in the refractive index of the cell), employing either phase contrast or interference microscopy. The interference microscope offers the greatest promise, but, since the necessary equipment was not available, initial studies were made with phase contrast equipment which was available.

In principle, the determination of the refractive index of a cell by phase contrast microscopy involves varying the refractive index of the medium

until a change in the phase contrast is observed. For example, in pure water substrate, a bacterial cell having a higher index of refraction will be seen in the phase contrast microscope as bright or dark (depending upon the particular optics), because of the difference in refractive indices of the water and the cell. If the index of refraction of the substrate is increased, so that it is greater than that of the cell, the contrast will shift from bright to dark, or dark to bright (again depending upon the particular optics). When the index of refraction of the cell and that of the substrate are equal, the cell is hardly visible. This method of determining an index of refraction has been employed for many years in the study of crystals and minerals, but the lack of a suitable substrate has until recently rendered it useless for the study of living cells. Barer and his colleagues[6] have now removed this objection by demonstrating that solutions of Bovine Albumin, Fraction V (AV) meet the exacting requirements of these studies.

Solutions of AV have many advantages: concentrations as great as 35 per cent are nontoxic to many cells; the AV does not permeate most living cells; the material is readily obtainable in a purified state,[†] either as a sterile aqueous solution or a dry powder; and the refractive index of solutions of AV can be easily determined by ordinary refractometry. Of special value is the fact that the molecules of AV are so large in size that the osmotic effect of AV solutions is small--each 10 per cent of AV concentration in water being

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[6] R. Barer, "Phase Contrast and Interference Microscopy in Cytology," Chap. 2, Vol. III (Cells and Tissues) Physical Techniques in Biological Research, Edited by Gerald Oster and Arthur W. Pollister, Academic Press, Inc., N. Y. (1956).

[†] Nutrional Biochemical Corporation, Cleveland 28, Ohio.

osmotically equivalent to a sodium chloride solution of less than 0.1 per cent. This special characteristic of AV solutions suggests the value of this material in determining the water-concentration response of living cells to varying osmotic differentials, and the applicability of this approach to the study of the water content of airborne bacterial cells. If no more direct method can be obtained, it is felt that the conditions of the aerial environment can be simulated by using solutions of AV in which the osmotic effect is made equal to that existing in the airborne particle by the addition of the proper concentration of other solutes. In this manner the protein concentration (and water concentration) of the bacterial cell could be determined and viability determinations could be made either on the basis of motility or reproductive ability.

Thus far it has been shown that the method of phase contrast reversal in AV solutions may be applied to the estimation of protein concentration. Methods have also been developed which can be applied to the study of protein and water concentrations of bacterial cells under conditions of varying osmotic differentials. The actual application of these methods is currently being attempted.

To verify the applicability of the method, the two available phase contrast microscopes were compared; one being a Bausch and Lomb, the other a Leitz. Although the high-dry objective of the Bausch and Lomb has a much greater working distance than does the Leitz, the greater convenience in using the adjustable substage condenser of the Leitz decided in favor of this instrument. Either instrument showed phase contrast reversal equally well when comparing a bacterial suspension in water (refractive index of 1.33) with one in a solution of AV having a refractive index of 1.41. An Abbe refractometer was employed to determine the refractive indices of the solutions.

In order to prevent AV solutions from evaporating while being studied, a sealed preparation was devised. The usual depression slides are useless in phase contrast work, and the available flat-bottomed counting chambers (such as the Dunn) are too thick for good phase contrast resolution. To overcome these objectional features slides were cut from sheets of methacrylated plastic 1.0 mm thick. Similar sections of plastic having 5/8-inch holes drilled out were cemented on the slides. The resulting slides thus have a flat-bottomed cavity 1.0 mm deep mounted on a slide 1.0 mm thick. Filling the cavity with AV solution and bacterial suspension and then mounting a coverslip over the cavity produces a sealed preparation which is thin enough for good phase contrast resolution. The relatively large volume contained by the cavity of such a slide is useful, because after phase contrast examination has been completed, the refractive index of the solution can be determined in the Abbe refractometer.

In order to determine the effect of AV solutions on the viability of Serratia marcescens (ATCC 274) various concentrations of AV were prepared and mixed with suspensions of the test organism and the preparations were sealed in the prepared slides. The final concentration of AV in these mixtures varied from 9.5 to 33 per cent. Immediate examination of these preparations with phase contrast showed phase reversal was exhibited in those having an AV concentration of 28 per cent or greater. Motility was observed only in those having an AV concentration of 20 per cent or less. It was later determined that reduction of motility in these preparations was due to increased viscosity of the solutions. All preparations were held at room temperature for 24 hours and

re-examined. It was observed that no change in phase contrast reversal occurred during the holding period, except for a few very large cells in the preparations of 28 per cent or greater. In these concentrations no phase reversal was observed after standing, indicating that these large cells had been infiltrated by the AV, and that the majority of the cells (of normal size) had not been infiltrated by the AV.

Further study of the sealed preparations of various concentrations of AV and S. marcescens which had been held for 24 hours showed that the majority of the cells had undergone division during this period. In the solutions of lower viscosity this was evidenced by increased numbers, high motility, increased pigmentation and odor production. In the preparations of higher viscosity, reproduction was evidenced as clumps of cells resulting from the division of original single cells. In these preparations the single cells which had not undergone division generally gave evidence of infiltration by the AV (no phase reversal in a preparation exhibiting reversal by the more normal cells).

On the basis of further studies similar to that described above, the following conclusions were reached: solution concentrations of AV as great as 33 per cent are not toxic to S. marcescens; the organism will grown and reproduce in these solutions; and the normal living cells of S. marcescens are not infiltrated by AV even on standing. For these reasons, it was concluded that the method of phase contrast reversal with AV solutions in sealed preparations is applicable to the estimation of the protein and water concentration of living cells of S. marcescens. The protein content of cells of a 44-hour beef broth

culture was determined to be approximately 26 per cent wt/wt by this method, using the formula

$$C = \frac{R.I. - 1.33}{0.0018}$$

where C is the concentration of protein, wt/vol; R.I. is the estimated refractive index of the cell; 1.33 is the refractive index of water; and 0.0018 is the increment of refractive index of protein, per cent wt/vol. The volume of 1.0 gm of protein is taken as 0.75 ml, and concentration, wt/wt, is computed. The concentration of water, wt/wt, is taken as the difference between that of the protein concentration, wt/wt, and 100 per cent. For the cells of the 44-hour culture of S. marcescens the water concentration was found to be 74 per cent.

Estimating the refractive index of bacterial cells by the above method is tedious and time-consuming. A number of preparations with varying concentrations of AV must be made and examined; the more preparations examined the more closely can the refractive index be estimated, because it is estimated as the value falling between the refractive indices of two preparatives, one of which shows the majority of the cells without phase reversal and the other in which the majority of the cells exhibit phase reversal. The interference microscope makes possible the same determination with the use of not more than two separate preparations, and it is hoped that such an instrument can be obtained for this work.

As a part of the search for a larger microorganism for these studies, cultures of Azotobacter agile have been initiated, and the protein content of

these cells estimated by the method described above. The cells of A. agile are large enough to permit a distinction to be made between the cell sap and the inclusion bodies. The cell sap showed a water content of approximately 77 per cent, but the inclusion bodies appeared to have a much lower water concentration, never showing complete phase reversal even in 38-per-cent AV solution. The cells of A. agile did not reproduce in AV solutions, but did exhibit motility. In solutions too viscous for motility, this characteristic could be demonstrated even after several hours if the solution were then diluted with water. It was concluded that this method would be applicable to the estimation of the water content of living cells of A. agile as well as to the living cells of S. marcescens. As previously indicated, however, the method is tedious and time-consuming so work is being held in abeyance until it is known whether or not an interference microscope can be obtained for this purpose.

III.

EFFECT OF PRIOR CONDITION ON THE AERIAL VIABILITY OF CULTURES OF *Serratia marcescens* (ATCC 274-RED-CARMINE)

Carrying out studies on the effect of various humidities on the survival of washed cells in the airborne state necessitates a certain amount of manipulation of these cells prior to aerial dispersion. The 44-hour culture in 0.3-per-cent beef extract borth (30° C) is filtered through a sterile millipore filter leaving the cells on the surface of the filter. The millipore filter is then washed thoroughly with sterile water. Following this procedure the filter is introduced into a measured amount of sterile water (usually equivalent to the volume of culture filtered) and broken up by vigorous agitation. This suspension is then strained through a coarse scintered glass filter to remove the particles of the millipore filter. The filtrate containing the washed cells is then place in the atomizer for aerial dispersion. Airborne cells dispersed in this manner exhibited a significantly greater aerial death rate (k) for intermediate humidities than cells dispersed directly from the beef extract culture. The question arose as to whether this difference was due to the unprotected condition of the washed cells, or whether it resulted from some insult occasioned by the manipulations of the cells prior to aerial dispersion.

Previous consideration of the state of cells prior to aerial dispersion indicated that culture "age" is a significant factor; cells from "young" cultures generally exhibited very high values of k. However, the results obtained with such cultures were so erratic that no quantitative data of value were tabulated. Experience showed that cultures in the late log phase of growth yielded the most consistent values of k for any given condition, and

most of our experimental work has been conducted with cultures of this type. However, consideration of the finding that washed cells are more adversely affected by the airborne state at intermediate relative humidities than are the cells dispersed from the culture without manipulation, suggested that culture "age" might be a factor in this phenomenon. That is to say, the process of transferring the cells to a new environment might well induce a change in the physiological condition of these cells, different from that of the cells dispersed without prior manipulation; it being assumed that cells in both instances are in the "resting" stage because of the storage of all suspensions in the water bath at 10° C during the atomization process.

In order to evaluate the effect of the various manipulations on the values of aerial die-away, cells handled in various ways were dispersed into the air under various conditions of relative humidity, and $\underline{k}$ values determined. In every case 44-hour cultures in 0.3-per-cent beef extract broth (30° C) were used as the source of cells. The control consisted of the original culture. For the purposes of the experiment washed cells were prepared as indicated previously, and dispersed into the air from water. To estimate the effect of the manipulations, cultures were filtered through millipore filters, the filtrate run through in the same procedure as employed with water, and finally recombined with the filter to yield a suspension of cells in the original beef broth. The recombined cells and culture filtrate were thus subjected to the same manipulative procedures as the suspension of washed cells. In order to evaluate the effect of the new suspending environment, cultures were filtered and washed with fresh beef extract broth, and resuspended in additional

fresh broth prior to atomization. The results of these studies are shown in Figure III. 1, where the values obtained for $\underline{k}$ are plotted for the various prior conditions of the cells according to the relative humidity at which these values were obtained. All observations were made at 20° C.

Examination of the information shown in Figure III. 1 shows that the manipulative processes are probably not responsible for the differences in $\underline{k}$ values obtained, except perhaps at 80-per-cent relative humidity where a decrease in $\underline{k}$ appears to result from the manipulations. However, the general shape of the three curves representing the data obtained from aqueous washed cells, original cultures, and the filtered and recombined cultures are distinctly similar. It is our interpretation that this similarity indicates that the manipulation of the cells during the filtration and reconstitution had little if any effect on the aerial viability, and that the increased values of $\underline{k}$ for the washed cells at intermediate humidities and decreased values of $\underline{k}$ at higher humidities were due to the differing responses of these cells to the aerial environment. However, these differences are minor compared to those shown between the above three conditions and those shown for the cultures filtered, washed and resuspended in fresh broth. In this case the peak of the curve is shifted towards the higher humidities, and the entire curve shows much lower values of $\underline{k}$ than any of the others shown in Figure III. 1. These differences are interpreted as further evidence that the manipulations had little if any effect on the values of $\underline{k}$ obtained.

Further consideration of the significance of the lower values of $\underline{k}$ exhibited by cells resuspended in fresh broth has not as yet yielded information which can be utilized in assigning a single causative factor to this phenomenon.

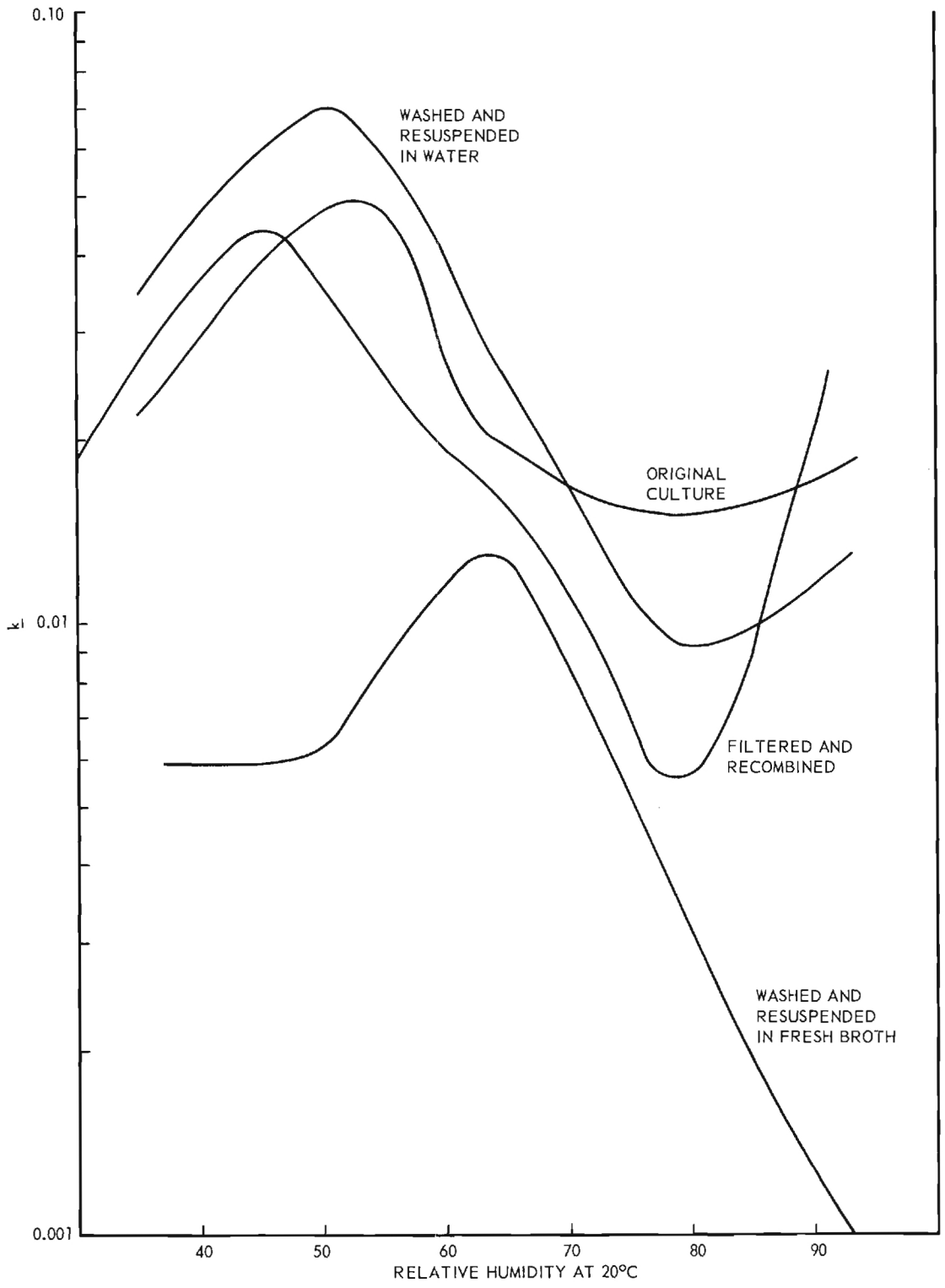

Figure III. 1. Effect of Prior Treatment on Aerial Viability of Serratia Marcescens.

Not all of the viable cells are recovered during the washing (some apparently adhere to the surface of the MF), but the possibility that some selection process occurs during washing seems slight since the filtered and reconstituted cells show response to the aerial insult similar to that of the original culture. Other factors--such as aeration, temperature, state of cell divisions and atomization procedures--were held identical for all cases. Complete exhaustion of the nutrient substrate seems doubtful, even if some rational relation between nutrient exhaustion and aerial death could be postulated. The greatest mass of bacterial protoplasm, living and dead, can be only something less than 1.0 mg/ml, and the stock broth contains 3.0 mg/ml of nutrient solids.

To date, the only factors which appear to offer an explanation of this phenomenon are the slight changes in pH (fresh broth, about 7.0, and 44-hour culture, about 8.0), and the possibility that some toxic substance accumulates in the culture (the removal of the toxic substance increasing the chance of survival of the cells when dispersed into the air). Further studies of these possibilities are planned.

The finding that manipulative procedures employed in changing the aqueous substrate of the bacterial cells does not per se effect a change in the response to the airborne state is of considerable value in elucidating the various factors which do bring about such changes. This approach is being actively utilized in current studies. However, it is felt that the beneficial change effected by resuspension in fresh broth is even more significant because of its possible bearing not only on the response of these cells to the aerial environment, but also because of the light that may be shed on the basic problem of

factors limiting the maximum numbers of bacterial cells obtainable in a culture under particular conditions. Although this is not in the mainstream of our current studies, it is hoped that some further investigations can be carried out in this direction.

IV.

CORRELATION BETWEEN GROWTH CURVES AND AERIAL DEATH RATE FOR WASHED CELLS OF *Serratia marcescens* (ATCC 274-RED-CARMINE)

In examining the results of experiments involving the increased recovery of viable airborne bacteria in sampling fluid enriched with brain-heart extract, the question of harmful effects short of death has been posed. The fact that simple sampling fluids yield appreciably lower numbers of viables, yet produce data which yield essentially the same values for aerial death (k) indicates that the airborne state produces some effect short of death. We have suggested that this difference is due to the presence of a sensitive segment of the airborne population; this segment is demonstrated in enriched fluid, but not in simple fluids. It was further suggested that the more hardy segment of the population is demonstrated by both fluids, and both the hardy and sensitive segments of the population are equally affected by the lethal effects of the airborne state.

Attempts to characterize the sensitive segment of the airborne population by comparing relative numbers of viables demonstrated in various sampling fluids have been unsuccessful. The ratio of recoveries, enriched to simple fluids, ranges from 1.3 to 6.5 and no correlation has yet been shown between total numbers (5,000 to 100,000/L), or die-away value, k (0.007 to 0.05), although the greatest ratios are usually associated with the smaller values of k. This finding suggests that even the enriched fluids are not demonstrating all of the viables in the sensitive segment of the airborne population, but approach complete demonstration when the value of the aerial death rate is lowest.

In order to approach the problem of characterizing the postulated sensitive segment of the airborne population, several other methods were considered. Manometric determination of respiratory activity was given considerable study, but discarded because (1) the difficulty of collecting sufficiently large numbers of airborne cells for accurate manometric determinations, and (2) the high endogenous activity of the cells results in forcing the interpretation of manometric studies into a simple model which yields results that by themselves are hardly more significant than those obtained from direct plating counts.

The direct microscopic observation of total numbers of cells and the numbers which will undergo single or multiple divisions appears to be the most effective method for characterizing the various segments of the airborne population. Efforts to develop such a technique appear to be promising. Although not sufficiently advanced to warrant immediate application, this method consists of collecting the airborne cells in the standard 1.0 L/min critical orifice - liquid impinger, filtering all or part of the fluid through a millipore filter, incubating the filter, and making microscopic examination of the number of singles, doubles, and other multiples as time of incubation progresses. Examination of a sufficiently large number of filters at various stages of incubation will yield information on the total initial number of cells collected, the relative number which are capable of initiating division, the relative number which are capable of producing various multiple divisions, and the time at which divisions are initiated. The accumulation of such data might warrant the examination of similar materials by a micromanometric technique, employing these data in the interpretation of the respiration data.

The direct study of growth responses of collected airborne organisms has proved fruitful and suggests the value of more extended studies in characterizing the various segments of the airborne population. The current studies were carried out with 44-hour cultures (0.3-per-cent beef extract broth, 30° C), filtered on millipore filters, washed with sterile de-ionized water, and resuspended in sterile de-ionized water, from which the organisms were dispersed into the air, under various conditions of temperature and humidity (to yield various values of $\underline{k}$). Washed cells were selected for this work because they appeared most sensitive to the airborne state. (See Section III of this report on effect of prior condition of cells on $\underline{k}$.) Samples of the airborne cells were collected in 1.0 L/min critical orifice - liquid impingers, employing enriched (BHI) fluid; the sampler fluid was subsampled and viable counts made by plating at regular intervals. The incubation temperature was 30° C.

Plotting the data obtained from growth studies on collected airborne cells produced curves of the types shown in Figure IV.1. In this figure the numbers of viables demonstrated after various intervals of incubation are plotted against (1) the time of incubation, (2) numbers as the logarithmic ordinates and (3) time as the arithmetic abscissae. Curves similar to plot A were obtained from collected airborne organisms which exhibited a low value of $\underline{k}$; plot B curves were from those exhibiting high values of $\underline{k}$. All curves were first analyzed graphically to determine L (lag time of Hinshelwood,[1] or T_L of Monod[2]),

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[1] C. N. Hinshelwood, The Chemical Kinetics of the Bacterial Cell. Clarendon Press, Oxford, 1946.

[2] J. Monod, "The Growth of Bacterial Cultures," Annual Review of Microbiology 3, (1949).

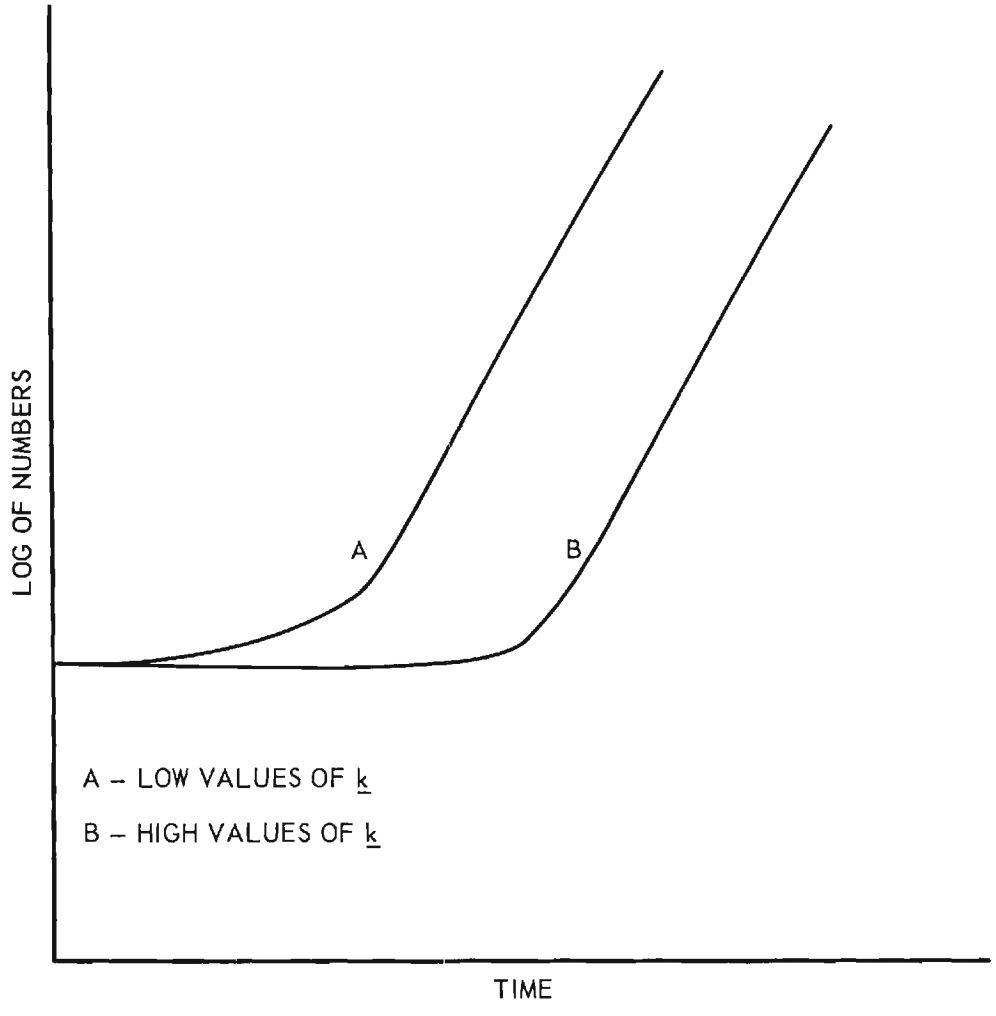

Figure IV. 1. The Two Types of Growth Curves Exhibited by Captured Airborne Cells of Serratia marcescens.

and G (generation time, g of Squires and Hartsell[3] or Finn),[4] as indicated in Figure IV.2. This analysis consists of extending the straight line formed by the logarithmic section of the growth curve, so that it intersects the horizontal line representing the numerical value of N_o (initial inoculum). The length of time between this intercept and zero time is L, which is a function of the lapse of time actually occurring before initiation of division (as contrasted to the idealized state where initiation of division would occur at zero time). The numerical value of G is the generation time, that is, the time required for any value of N to become (2) (N), in the exponential phase of growth. In Figure IV. 2 G is most easily estimated by dropping a line from the point (2) (N_o) to the line representing the numerical value of N_o, and G is therefore the time lapse represented between the two intercepts. Because all of the studies herein reported were carried out with essentially the same number of viables in the initial inoculum, no consideration was given to size of inoculum. Presumably, variations in size of original inoculum would have required the plotting of N/N_o instead of N.

The estimation of values for L and for G from growth curves provides certain information about the bacterial population involved. Values for G indicate the time required for the viable cell population to double in number. The relative size of the value for L indicates the overall lag, prior to the initiation of division in the majority of the cells, but does not yield any

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[3] R. W. Squires, and S. E. Hartsell, "Measurement of Relative Lag Time," Journal of Bacteriology 69, 226 (1955).

[4] R. K. Finn, "Measurement of Lag," Journal of Bacteriology 70, 352 (1955).

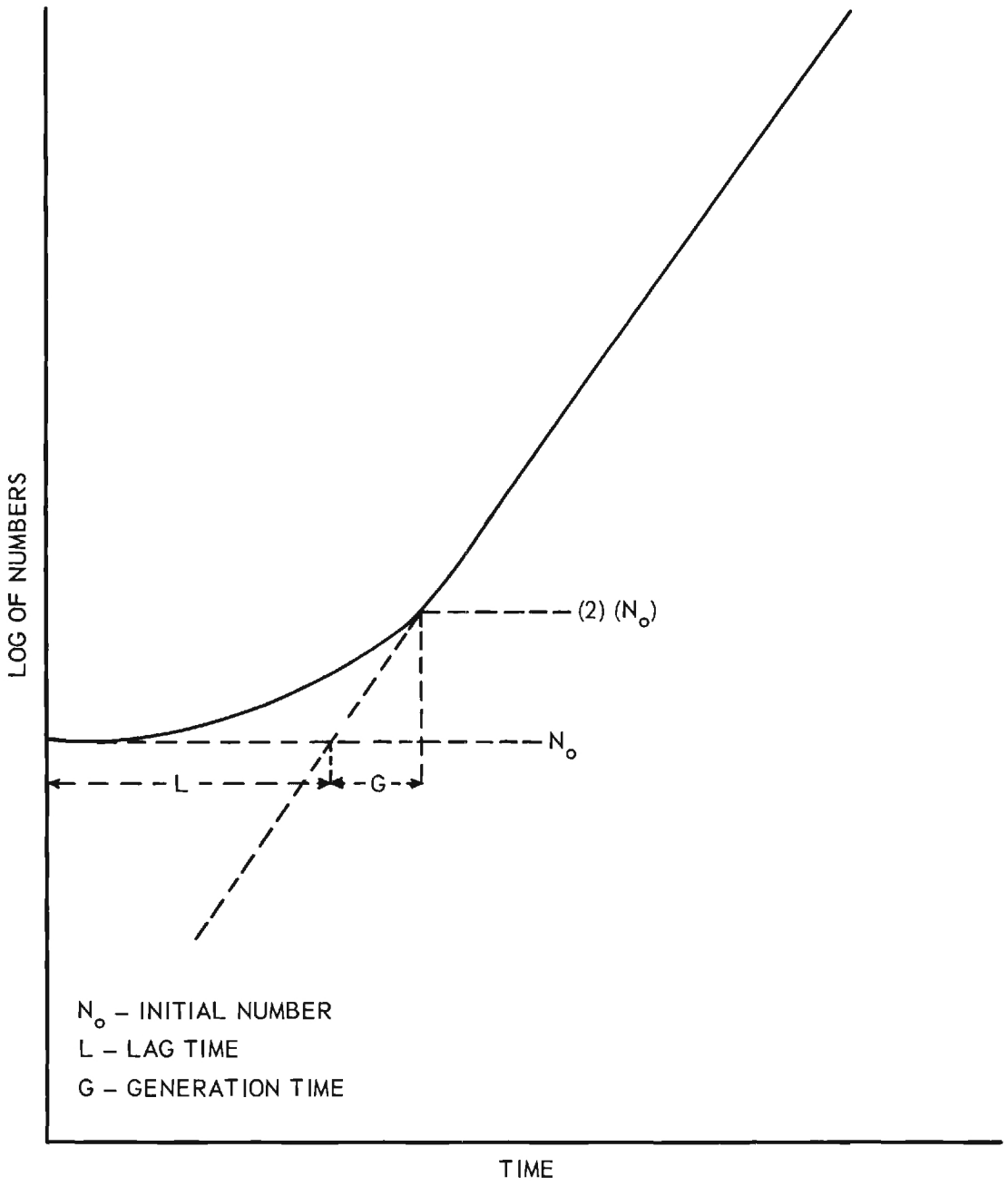

Figure IV. 2. Graphical Analysis of Growth Curves for Estimation of Lag Time and Generation Time.

information as to the rate of this initiation. Examination of growth curves of Types A and B (Figure IV.1) showed that division was not being initiated at the same rate in both instances. Initiation of division progressed at a steady rate in Type A but in Type B was delayed (initial stationary phase) before progressing rapidly. Critical analysis of the growth curves showed that an exact mathematical description of the length of the initial stationary phase exceeded the exactness of the data available. However, stepwise analysis of the number of cells dividing at any time during the early part of the growth curve showed that any estimate of the time at which a significant portion of the original cells began to divide would yield a useful estimate of the length (in time) of the initial stationary phase. Further examination of the experimental data showed that a 30-per-cent increase was the least increase that could be estimated with any degree of confidence.

On the basis that a 30-per-cent increase in the value of N_o could be determined with confidence from a graphical analysis, the length of time required for the production of (1.3) (N_o) cells was estimated, as shown in Figure IV. 3. This value, Z is corrected for the actual time lapse between initiation and completion of division which is the value for G, so that Z minus G yields a value for S, which is a relative estimate of the length (in time) of the initial stationary phase. Values of S, L, and G are shown in Table IV. 1, with the corresponding values of $\underline{k}$.

Examination of the data tabulated in Table IV. 1 indicates a reasonable degree of correlation between increasing values of $\underline{k}$, L and S for the collected airborne cells. Because L is a measure of the overall lag of the growth curve

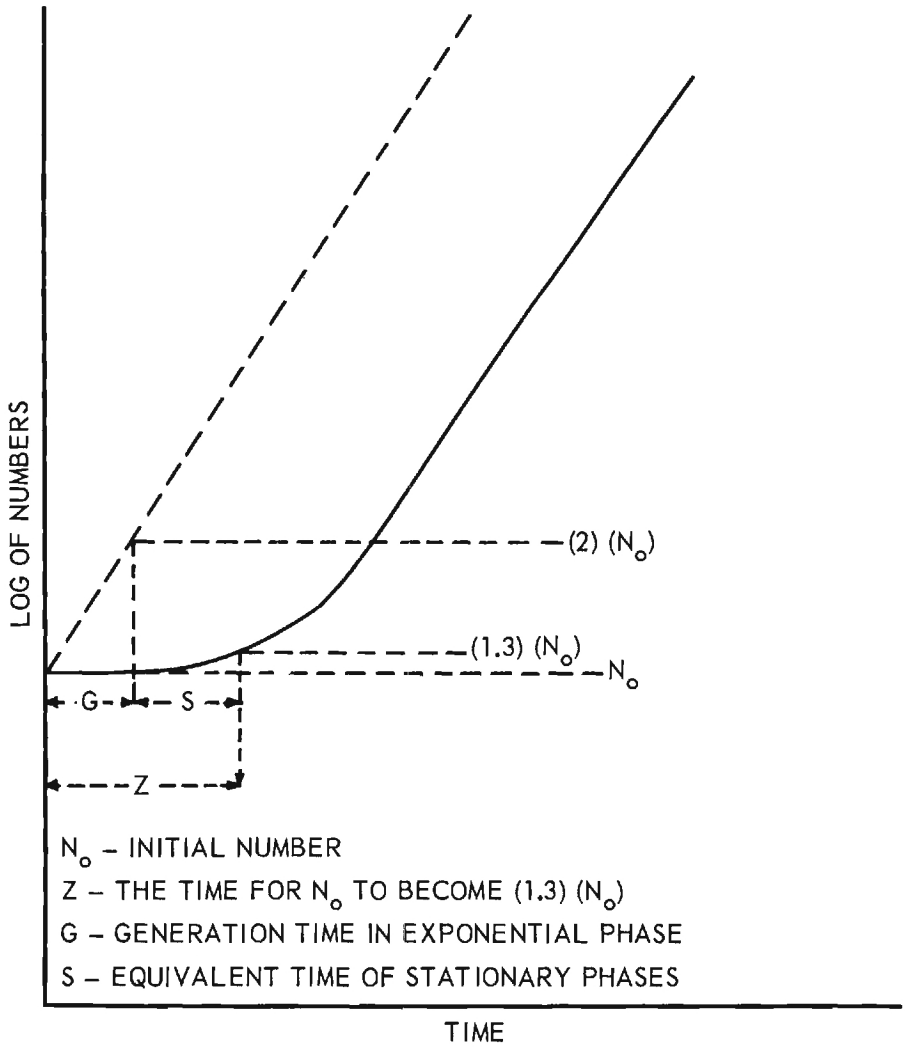

Figure IV. 3. Graphical Analysis of Growth Curves for Estimation of Length of Time of Initial Stationary Phase.

TABLE IV-1

TABULATION OF GROWTH CURVES, OF WASHED CELLS, FOLLOWING COLLECTION AT VARIOUS VALUES OF k

k	L	S	G
0.02	1.95	0.90	0.55
0.02	1.90	0.75	0.55
0.02	2.00	0.80	0.50
0.03	1.85	1.20	0.50
0.03	2.00	1.45	0.55
0.03	2.05	1.25	0.50
0.04	2.25	1.80	0.50
0.04	2.25	1.60	0.55
0.06	2.40	1.90	0.45
0.06	2.40	1.85	0.45
0.06	2.50	1.90	0.45
0.06	2.50	1.85	0.50
Controls			
Control	1.65	1.30	0.55
Control, No Dow AF†	2.00	1.55	0.50

L = time lapse in hours between actual curve and curve if there had been no lag.

G = generation time, in hours.

S = time lapse prior to initiation of division of 30 per cent of the cells--equal to time for $N/N_o = 1.3$, minus G. Equivalent to initial stationary phase.

†All other samples contained one loopful of Dow AF, antifoaming agent.

this correlation indicates that increasing values of k result in an increasing suppression of the initiation of division in the majority of these cells. This is consistent with the observation of Berendt[5], who has stated that during incubation there was an increase in the average length of aerosolized cells, as compared to that of similar cells prior to atomization. However, S is a measure of the length of the initial stationary phase, and the relative smallness of the values of S exhibited at lower values of k indicates a stimulation of the initiation of division among some of these cells, especially when compared to the S values for the controls. This type of response is not dissimilar from that observed by Squires and Hartsell[6] in freezing and thawing bacterial suspensions, where certain conditions stimulated the initiation of division.

Because of the involved nature of these responses short of death to the airborne state, a critical interpretation was not attempted at this time. However, the results obtained thus far certainly show the changes short of death are induced in airborne bacteria, and that some of these changes are reflected in the growth curve responses. These findings warrant the further investigation of these changes by more detailed studies of the actual growth, and if these indicate the need, by respiration studies.

It is to be hoped that a more quantitative description of the sensitive segment of an airborne bacterial population will lead to a better understanding of certain other anomolous results obtained in sampling such populations,

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[5] R. F. Berendt, "Some Physiological and Immunological Properties of Aerosolized Pasteurella pestis," Symposium on Microbial Aerosols and Respiratory Infections, Society of American Bacteriologists, 1957.

[6] R. W. Squires and S. E. Hartsell, "Survival and Growth Initiation of Defrosted Escherichia coli Affected by Frozen Storage Menstrua," Applied Microbiology 3, 40 (1955).

and will be of value in assaying the probability that any airborne microorganism will have the capacity to initiate growth under any given set of conditions, such as when inhaled by human susceptibles.

V.

STUDIES ON THE SURFACE AND WATER CHARACTERISTICS OF AGAR PLATES

Analysis of previous studies has posed the question as to whether the problems involved in recovering and demonstrating viable airborne bacteria under various conditions of temperature and relative humidity are primarily those related to the sampler or whether they are primarily related to the nature of the bacterial particle. Because of the greater variation in results obtained with agar plates, as compared with liquid samplers[†] it was tentatively postulated that the characteristics of the agar surface varied with varying temperature and humidity. In order to test this hypothesis a number of studies have been carried out on the surface and water characteristics of agar plates.

Attempts to determine the presence or absence of a measurable film of water at the agar surface by conductivity measurements were apparently unsuccessful. However, one of the equipment setups devised for this purpose did prove useful in measuring the rate of shrinkage of the agar. In this instance the edges of the platinized sensing electrodes were positioned just above the surface of the agar by means of a screw micrometer. Conductance was measured with an Industrial Instruments[††] Model RC bridge. The electrodes were lowered until a measurable conductance was obtained; this was found to be a steady value, regardless of the apparent condition of the surface of the agar. Because

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[†] T. W. Kethley, E. L. Fincher, and W. B. Cown, "The Effect of Sampling Method Upon the Apparent Response of Airborne Bacteria to Temperature and Relative Humidity," J. of Infectious Diseases 100, 97-102 (1957).

[††] Industrial Instruments, Inc., Jersey City 5, N. J.

of the prior bias that the water film would change, these results were considered of doubtful value. In following the conductance over prolonged periods of time, however, it was found that the electrodes had to be lowered between measurements. Examination of the records indicated that the micrometer readings could be converted into values for the shrinkage of the agar slab. Thus, for a plate (100-mm petri dish) of nutrient agar (1.5-per-cent agar) at 55-per-cent relative humidity, (76° F), the shrinkage was 0.18 mm/hr. Comparing this to the actual weight loss of the plate during the exposure, it was found that the agar shrinks at a slightly greater rate than is indicated by assuming the weight loss to be equivalent to volumetric loss.

It was not determined whether or not the actual shrinkage is significantly greater than the equivalent volumetric change indicated by weight loss (if true, this would signify a decrease in volume due to change in the agar matrix), but it was felt that this finding indicated that any film of water originally present on the surface of the agar continued to exist over a long period of time. This suggestion was contrary to the original hypothesis; because of this it was felt necessary to prove or disprove the continued presence of the film.

Starting with the model which assumes a measurable film of water on the surface of the freshly prepared agar, the most direct evaluation of the continued existence of this film would be in terms of water loss. That is, if water loss from the surface is at a steady rate, equal essentially to that of pure water under the same circumstances, then the surface film of water is constantly being renewed. Initial comparison of the weight loss from exposed dishes of water and nutrient agar showed this to be true. In still air at

65-per-cent relative humidity, 68° F, the evaporative losses from nutrient agar and from water were both consistently 14 x 10^{-6} gm/cm^2/min/mm Hg, for periods of time up to 24 hours (78.5 cm^2 exposed surface). Observations were discontinued at the end of 24 hours because the agar in the dishes often shrank and cracked at this time of exposure. The Langmuir equation for evaporation from a surface in vacuo was employed to determine the theoretical loss:

$$\frac{dm}{dt} = 3/13\ P\ \sqrt{3M/RT}$$

where dm/dt is in gm/cm^2/sec; P is the vapor pressure, dynes/cm^2; R is the gas constant, ergs/mole; T is the absolute temperature; M is the molecular weight. The actual value of P was determined as the difference between the vapor pressure over the saturated water surface at the wet bulb temperature, and that of the water vapor in the ambient air. For the conditions under study, the theoretical loss should be 15 x 10^{-6} gm/cm^2/min/mm Hg for water. This value is slightly greater than that experimentally observed because no correction was made for the stagnation existing above a film of water in a dish of the type employed. The agreement between the observed facts and theoretical calculations was taken as ample evidence that no great error had been incurred in the experiment. It was therefore concluded that under the conditions of the experiment the rate of water loss from the surface of nutrient agar is not different from the rate of water loss from the surface of pure water for a period of 24 hours, and, if a measurable film of water initially exists on the surface of the agar, this film must continue to exist for the 24-hour period of exposure.

At this point in the investigations the depth of the initial surface film on agar plates was given consideration. It is a general observation, in our own and other laboratories, that agar plates seem to require "ageing" before use; otherwise discrete surface colonies of bacteria cannot be formed. Analysis of the procedure followed in the preparation of dishes of agar for settling plates showed that this "ageing" process is most probably the time lapse required for the droplets of water adherent to the inner surface of the lids of dishes to be taken up by the agar mass. In every instance an agar plate which was "aged" sufficiently to permit discrete colony formation was found to be a plate having a dry surface on the inner lid. This was verified by pouring plates, allowing the agar to cool and set firm, then removing the original lid and replacing it with a dry one. Such plates were found to be satisfactory for the demonstration of discrete colonies within a period of less than one hour. This is in contrast to our usual procedure of preparing settling plates the day prior to use, and indicates that a distinct pool of water must collect on the agar surface whenever the lid above has drops of free water. This finding is also interpreted to mean that some minimum thickness of water film exists whenever the agar contains the only free water in the closed system represented by the dish with a lid over it.

In order to examine the rate of loss of water from agar surfaces more exactly, measurements were made under conditions of turbulent air flow. For this purpose, the dishes were placed in the bottom of a 4-inch diameter cylinder having a diffuser above the plate. Operating at 68° F (20° C), air with a dew point of 30° F was passed over the plates at 1.0 cfm. The dew point of

the air exiting from the system was measured with a previously calibrated Gow-Mac[†] Model RCCT thermal conductivity cell and recorded with a General Electric[††] self-balancing potentiometer and Esterline-Angus[†††] Model AW milliammeter recorder. This system permitted the exact, minute-by-minute recording of the water loss from an exposed plate, as indicated by variations in the dew point of the exit air.

A study made of the rate of water loss from plates of 1.5-per-cent nutrient agar exposed to dry air at an air flow of 1 cfm in a 4-inch cylinder showed that the loss is identical for the first 20 minutes of exposure to that incurred by a plate of pure water under the same circumstances. After 20 minutes the rate of loss from the agar surface fell to about 80 per cent of that from the pure water. It was assumed that at this point a "skin" must form on the surface of the agar, slowing down the diffusion of water to the surface. The actual evaporative losses during the first 20 minutes of exposure were at the rate of 320×10^{-6} gm/cm^2/min/mm Hg. This is some 23 times as great as that observed for still air evaporation.

Since information obtained thus far indicated that agar might form a surface skin if evaporation was forced to proceed at a sufficiently rapid rate, attempts were made to evaluate this skin. The problem was approached on the assumption that a skin at the surface would offer greater resistance to penetration than would otherwise be exhibited. The most sensitive indication of

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[†]Gow-Mac Instrument Co., Newark, N. J.

[††]General Electric Company, Schenectady, N. Y.

[†††]Esterline-Angus Company, Inc., Indianapolis, Ind.

penetration resistance is obtained when the force required to penetrate is measured. A variety of tools and measuring devices were utilized, the most satisfactory being a Shore† Durometer Type 00, in which the original tool was replaced by a 1/8-inch diameter steel ball. With the Durometer a definite surface penetration value could be obtained even with freshly prepared agar slabs, provided the agar concentration was as great as 1.5 per cent. For lower concentrations of agar the surface offered no resistance to penetration by the steel ball. Typical values are shown in Table V. 1. These results indicate that the method is not measuring surface resistance alone. This is shown by the increase in Durometer readings with increasing concentration of agar, and with increasing time of exposure, these increases in reading being of the same order as might be expected to result from increasing agar concentration due to evaporative losses (that is, the 72-hour exposure of 1.5-per-cent agar at 45-per-cent relative humidity represents a loss of water from a 1.5-cm depth slab which results in a 2.0-per-cent agar slab 1.0 cm in depth). However, the response of the instrument during the taking of a reading definitely indicates the presence of a resisting skin at the surface of 1.5- and 2.0-per-cent agar slabs. Unfortunately, this has not been verified by any other method, and the Durometer testing has been of little value in advancing our knowledge of the exact condition of the agar surface. On the other hand, because the test does appear to indicate changes in agar concentration, this procedure has been applied to the testing of stored agar plates in order to determine rapidly and simply whether or not significant evaporation has occurred during storage.

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†Shore Instrument and Mfg. Co., Jamaica, N. Y.

TABLE V. 1

VARIATION OF DUROMETER READINGS WITH VARIATION IN CONDITION OF AGAR

Condition of Agar	Durometer Readings for Various Agar Concentrations		
	1.0 Per Cent Agar	1.5 Per Cent Agar	2.0 Per Cent Agar
Freshly prepared	0	5	25
Closed, 7 days old	0	5	25
Open 24 hrs 65% RH	-	10	35
Open 72 hours 65% RH	5	15	45
Open 72 hours 45% RH	5	25	>100

At this point in the investigations on the character of the agar surface it was felt that the working model which assumes a film of free water which exists for some measurable period of time had neither been proved nor disproved with any degree of certainty. The fact that water diffuses freely from the agar matrix certainly indicates that any initial film of water will be maintained until some change in diffusion occurs. The fact that the rate of evaporative losses does decrease at the end of a definite period of time, depending upon the total evaporative loss, indicates that some change in diffusion actually occurs. However, failure to demonstrate the presence of a surface film which might account for the decrease in diffusion rate makes it difficult to envision the cause. The concentrations of agar attained even after prolonged evaporation are insufficient to account for this difference as being due to changes in vapor pressure over the agar. The failure to demonstrate a surface film suggests that the change in rate of evaporation might be due to changes

in the agar matrix. The possibly greater rates of shrinkage than acccounted for by volumetric changes occasioned by evaporation support this, as does the observation that the surface of 1.0-per-cent agar will not permit the formation of discrete colonies under any circumstances. However, our main concern was to determine whether or not changes do occur at the surface of an agar plate which might account for differences in sampling between agar plates and liquid samplers. For this reason, investigation of possible changes in the agar matrix was set aside and efforts were continued to measure the surface film of water.

The presence of free water can be determined by any number of multiple-step reactions requiring free water for completion which yield a colored end product. In applying this approach, a study was made of suitable carriers for such a reaction to indicate the presence or absence of free water at an agar surface. A variety of materials were examined and it was found that inexpensive lens paper[†] is the most nearly suitable of all the available materials. This lens paper is highly absorbent, and very thin, being made up of a few layers of fibers, forming a mat about 40 microns thick. When cut into strips about 5 x 0.5 cm the paper possesses a certain amount of stiffness which enables contact to be forced between the paper and an agar surface by holding only one end of the strip. When these strips were tested on agar surfaces it was found that any free water immediately infiltrated the paper and gave it a transparent appearance. Thus, testing for free water with strips of this lens paper required no added chemical system. When a strip is touched to a surface having

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[†] "Bioloid" Brand No. 17466, Will Corporation, Atlanta 1, Georgia.

a film of free water the paper immediately wets and becomes transparent. Similarly, an agar surface having only a trace of free water requires that the paper strip be pressed upon it, and wetting is gradual. For a dry agar surface, the paper can be pressed upon it and wetting will not take place for several seconds.

Employing strips of lens paper to detect the presence of measurable film of free water on agar surfaces yielded results which are consistent with our other findings--in still air, even when exposed to very low humidities, 1.0-, 1.5-, and 2.0-per-cent agar slabs will possess and maintain a film of free water for many hours; the 1.0-per-cent agar for at least 3 days, the 1.5- and 2.0-per-cent agar for at least one day. Considering all the evidence thus far accumulated we feel that there is ample proof that the surfaces of the agar plates which have been used in our studies for the collection of airborne bacteria are, in all instances, covered with a measurable film of free water, because none of the exposure times have ever exceeded a few minutes in still air. This conclusion is a direct contradiction of that previously offered by us to explain the differences between values obtained with settling plates and those obtained with liquid samplers under various conditions of temperature and relative humidity.[†] It is our present feeling that this fact places a greater significance upon the physical state of the airborne bacterial particle, changes in the physical state being the most likely source of an explanation of the observed differences if they are not caused by changes at the agar surface.

[†] T. W. Kethley, E. L. Fincher, and W. B. Cown, "The Effect of Sampling Method Upon the Apparent Response of Airborne Bacteria to Temperature and Relative Humidity," J. of Infectious Diseases 100, 97-102 (1957).

VI.

CHROMOGENIC VARIANTS AND CULTURE MEDIA

Synthetic Media and Amino Acid Additives.- The standard test organism remains a chromogenic variant, designated as R, which is isolated from Serratia marcescens (ATCC 274) cultivated on nutrient agar at 20° C. The R variant is removed from the agar surface and transferred to 0.3-per-cent beef extract broth (30° C). At intervals of 45 to 48 hours the R variant is transferred to a fresh broth medium. The constitution of a stable R-type culture has been found necessary for consistent results in aerial viability testing. Although the undifferentiated S. marcescens (ATCC 274) as received from the American Type Culture Collection† exhibits the standard viability response under the standard atmospheric conditions, continued serial transfer in 0.3-per-cent beef extract broth (30° C) has resulted in a deteriorated response under aerial test conditions. Concomitant cultural changes have been thus far related to the emergence of a wide range of morphologic and dimensional cell types[1].

The undifferentiated culture of S. marcescens (ATCC 274) containing the chromogenic variants designated as blood-red (BR), red (R), pink (P), and white (W) and cultured in 0.3-per-cent beef extract broth (30° C) will show a gradual shift in the distribution of color variants toward a predominance of the BR type (see Table VI-1). As will be shown later, there appears to be no selective viability among the various chromogenic types when exposed in the aerial state. However, the shift in the undifferentiated culture toward

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[1] E. L. Fincher, T. W. Kethley, and W. B. Cown, "Variations in the Aerial Viability Associated with Variation in Morphology of Color Variants of Serratia marcescens," Applied Microbiology 5, 131-135 (1957).

† 2029 M Street, N. W., Washington 6, D. C.

TABLE VI-1

CHROMOGENIC VARIANT DISTRIBUTION DEVELOPING IN 0.3-PER-CENT BEEF EXTRACT BROTH AND IN BUNTING'S MEDIUM

Beef Extract Broth		Variant Distribution - Per Cent Variant				
Culture Age† (Hr)	Viable Cells/Ml	BR	R	P	W	Total Colonies
0	448	18.4	72.4	9.2	0	217
2	595	22.0	68.4	9.6	0	376
4	379×10^1	17.4	75.7	6.9	0	887
6	503×10^2	20.0	73.0	7.0	0	1130
8	577×10^3	21.4	70.0	8.6	0	903
24	192×10^6	46.8	47.3	5.3	1	771
30	306×10^6	49.5	45.0	5.5	0	853
53	195×10^7	67.0	31.0	2.0	0	1238
149	61×10^7	89.0	10.0	1.0	0	317
Bunting's Medium						
0	541	23.0	67.0	9.0	1	432
2	661	17.0	73.0	9.0	1	593
4	224×10^1	18.0	73.0	8.0	1	1188
6	Not made					
8	193×10^3	21.5	71.3	7.2	0	195
24	109×10^6	25.0	67.0	7.0	1	559
30	50×10^7	19.6	70.6	9.8	0	276

(Continued)

TABLE VI-1 (Continued)

CHROMOGENIC VARIANT DISTRIBUTION DEVELOPING IN 0.3-PER-CENT BEEF EXTRACT BROTH AND IN BUNTING'S MEDIUM

Beef Extract Broth		Variant Distribution - Per Cent Variant				
Culture Age† (Hr)	Viable Cells/Ml	BR	R	P	W	Total Colonies
53	209×10^7	18.5	70.5	10.0	1	611
149	126×10^7	18.8	66.7	14.5	0	617
Original Inoculum						
---	---------	16.0	73.8	9.2	1	428

†Incubation Temperature: 30° C.

BR-type predominance will eventually result in a culture exhibiting an increased viability loss (k) in the airborne state.

Efforts toward the control of cultures maintaining a constant distribution of the constituent chromogenic variants were directed to the possible role of various amino acid types added to Bunting's synthetic culture medium (Table VI-2). This medium provides carbon and nitrogen sources in ammonium citrate and glycerol and provides an excellent culture medium for the demonstration of the various chromogenic types. A concentration of 0.5 mg/ml of the various amino acids was added to 100 ml of this medium.

Reconstitution of an inoculum culture having the original ratios of chromogenic variants (BR 17 per cent, R 59 per cent, P 22 per cent, W 2 per cent) was obtained by the selective removal of variant colony types from a 48-hour surface

TABLE VI-2

ANALYSIS OF BUNTING'S MEDIUM AND BEEF EXTRACT

Bunting's Medium for Differentiation of Color Variants:[†]

Glycerol	5.0 gm
Ammonium citrate	5.0 gm
$K_2HPO_4 \cdot 3\ H_2O$	10.0 gm
$MgSO_4$	0.5 gm
NaCl	0.5 gm
Ferric ammonium citrate	0.05 gm
H_2O, distilled	1000 ml
Agar	1.5%
pH 6.9	

Beef Extract - Typical Analysis:[††]

Total solids	80.0%
Ash in total solids	19.8%
Chlorides as NaCl	4.05%
Total nitrogen	8.67%
Ammonia nitrogen	0.16%
Peptone nitrogen	2.3%
Proteose nitrogen	1.2%
Total meat base nitrogen	5.05%
Nitrates	None
Tyrosine	None
Cystine	0.13%
Creatine	1.5%
Creatinine	1.5%
Iron	Nil

[†]Mary I. Bunting, "A Description of Some Color Variants Produced by Serratia marcescens, strain 274," J. Bacteriol. 40, 57-68 (1940).

[††]Courtesy of Dr. H. W. Schoenlein, Difco Laboratories, Inc., 920 Henry Street, Detroit 1, Michigan.

growth on Bunting's synthetic agar medium (20° C). Selected variant colony types in the ratios of 1:6:2:0 were thoroughly mixed and from the mixture an approximately 1.5-mm "bead" was transferred to 100 ml of sterile deionized

water. The amount of the inoculum used in the amino acid - Bunting medium was one milliliter of a 10^4 dilution in sterile deionized water added to 100 ml of culture medium and incubated at 30° C. Demonstration of the various chromogenic types was made from streaked plates of Bunting's agar medium incubated at 20° C. for 72 hours. Each plate was streaked from two drops of a 10^6 aqueous dilution of the original culture thus affording a quantitative estimate of the cell density in each amino acid medium. These estimates of cell density are relatable because the original inoculum was equal for all test cultures.

The resulting percentage incidence of BR and R types of chromogenic variants shows a distinct difference among the various amino acid additive media (Table VI-3). The relative percentage occurrences of the variant types show a grouping relationship under the amino acid types. It is of interest to note that, excluding the value of dl-serine, the total number of BR-type variants is the same (290 ± 42) irrespective of the total cell count. The relative percentage incidence of the BR-type is determined by the greater variation shown among the R-type variant colonies and to a lesser extent among the P-type variant colonies. The amino acid dl-serine is in a position of disagreement with the scheme of amino acid grouping, the much lower relative growth indicating that the original inoculum changed comparatively little during the growth period. Average values (dl-serine omitted) show the relative total growth as related to the amino acid groups (Table VI-4). The anticipated shift toward predominance of the BR type with culture age is shown in some of the selected cultures (Table VI-5) after 6 days of growth, the shift occurring in the control and in dl-methionine. It is notable that there is a stable distribution of variant types with a significantly great increase in total numbers in the cysteine medium.

TABLE VI-3

VARIANT DISTRIBUTION IN SYNTEHTIC MEDIUM
WITH ADDITION OF SELECTED AMINO ACIDS

		Variant Distribution - 48-hr Growth (30° C)								
		BR		R		P		W		Total
Amino Acid Group	Amino Acid	(No.)	(%)	(No.)	(%)	(No.)	(%)	(No.)	(%)	Colonies
Monoamino-monocarboxylic	cysteine, HCl	299	37.0	473	59.0	25	3.0	2	1	799
Monoamino-monocarboxylic	dl-methionine	300	32.0	578	62.0	51	5.0	1	1	930
Monoamino-monocarboxylic	dl-valine	259	27.7	637	68	41	4.3	0	0	937
	control	379	26.0	957	68.0	71	5.0	1	1	1408
Heterocyclic	l-hydroxyproline	288	26.0	688	64.0	98	9.0	7	1	1081
Heterocyclic	l-proline	286	26.0	729	67.0	68	6.0	2	1	1085
Heterocyclic	dl-tryptophane	236	24.0	705	70.0	53	5.0	1	1	995
Diamino-monocarboxylic	l(+)-arginine, hcl	251	22.0	783	70	92	8.0	0	0	1126
Monoamino-monocarboxylic	dl-serine	53	20.0	201	75.0	10	4.0	2	1	266
Diamino-monocarboxylic	l-asparagine	318	19.0	1242	73.0	127	7.0	6	1	1693
Initial	Inoculum	173	21.8	552	69.8	67	8.4	0	0	792

These studies are to be continued with an investigation of the effects of 48-hour interval transfers (30° C), maintaining the cultures in a condition of rapid increase. Further, these cultures should be tested for aerial viability

TABLE VI-4

AVERAGE DISTRIBUTION OF CHROMOGENIC VARIANTS
AND TOTAL GROWTH RELATED TO AMINO ACID GROUPS

Amino Acid Group	Average - Per Cent Variants				Total Colonies
	BR	R	P	W	
Monoamino-monocarboxylic	32.4	63.1	4.3	<1	888
Heterocyclic	25.0	67.0	7.0	1	1050
Diamino-monocarboxylic	20.0	71.0	8.0	<1	1409

under the standard conditions of atmosphere (68° F, 65-per-cent relative humidity). It will be necessary to filter the cells from the medium under study and resuspend them in 0.3-per-cent beef extract broth to provide a common dispersion substrate for the airborne state, or else to study them as washed cells in the aerial state.

Nutrient Agar Medium and Chromogenic Variants.- Since the chromogenic variants shift toward the BR type occurs rapidly in a liquid type medium, a study of the variant distribution was followed on nutrient agar slants at 30° C, transfers being made at intervals of 48 hours. The original inoculum was made from a sample culture as received from the American Type Culture Collection. The results are shown in Table VI-6. The variant type distribution was obtained from Bunting's medium at 20° C. A small "bead" of growth was removed from the nutrient agar surface and diluted to 10^6 in sterile deionized water, two drops of this dilution being streaked over the surface of prepared plates.

TABLE VI-5

VARIANT DISTRIBUTION OF SELECTED CULTURES AFTER 6 DAYS GROWTH (30° C)

Amino Acid	Variant Distribution BR (No.)	BR (%)	R (No.)	R (%)	P (No.)	P (%)	W (No.)	W (%)	Total Colonies
Cysteine, HCl	468	32.1	803	54.7	179	12.2	14	1	1464
Control	571	41.4	671	48.4	128	9.2	8	1	1378
dl-Methionine	396	40.5	339	35	231	23.5	2	1	968
l-Asparagine	263	21.7	738	60.3	208	17.0	8	1	1219

TABLE VI-6

VARIANT DISTRIBUTION FROM NUTRIENT AGAR SLANTS (30° C), TRANSFERRED AT 48-HOUR INTERVALS

Date	Chromogenic Types - Per Cent BR	R	P	W	Total Colonies
9/25/57	26.0	64	9.0	1	500
10/31/57	32.7	63.6	3.7	0	1322
11/7/57	36.3	59	4.7	0	591
11/12/57	48.8	46.7	4.5	0	88
11/19/57	55.3	40.5	3.2	1	749
Culture as Rec'd from ATCC					
9/16/57	17	59	22	2	835

Consideration was given to the effect of temperature on (1) the nutrient agar slant culture and (2) the pigment development of the chromogenic variant

colonies on Bunting's medium. From 72-hour nutrient agar slant cultures grown at different temperatures, Bunting medium streaked plates were prepared as outlined above and incubated at various temperatures. The results are shown in Table VI-7. During the course of making the observations a greater tendency was noted of BR-type emergence as related to the degree of "wetness" of the nutrient agar slant--excess moisture seeming to accelerate the BR-type emergence.

TABLE VI-7

EFFECT OF TEMPERATURE ON VARIANT DISTRIBUTION ON NUTRIENT AGAR AND VARIANT DIFFERENTIATION ON BUNTING'S MEDIUM

Culture Age	Source Culture Temperature	Incub. Temp. (°C) Streaked Plates†	Variants - Per Cent				Total Colonies	Pigmentation of Source Culture	
			BR	R	P	W		3 days	7 days††
(Hr)	(°C)								
72	20	30	45.7	48	5.3	1	672	orange-red	red
		20	45	50	4.0	1	636		
72	30	30	55.3	40.5	3.2	1	749	lavender-red	lavender
		20	49.2	46.5	3.3	1	858		
72	37	30	56	41	3	0	638	lavender-hue pink	purple
		20	55.5	40.3	3.2	1	641		
		Original inoculum							
			43	47	9	1	142		

†The results of 37° C for streaked plates were not tabulated because of the poor color differentiation on Bunting's agar medium. The colonies pigmented (lavender-hued) but did not form the characteristic BR, R, and P types.

††The 7-day old cultures were removed from the temperatures 20°, 30°, and 37° C at the end of 3 days and stored at 30° C for the succeeding period of 7 days.

Aerial Viability of Cells Derived from (1) Nutrient Agar Surface and (2) Beef Extract Broth; Differential Viability of the Chromogenic Variants.- It is of interest to know something of the relative aerial response of cultures derived from solid surface growth and from a liquid culture. Solid surface growth was harvested from the nutrient agar surface of the medium contained in a square-shaped one-quart milk bottle, solidification of the agar occurring while the bottle was lying on a side. Several drops of an aqueous culture suspension were distributed over the entire agar surface and incubated. Cells from the nutrient agar surface were gently harvested with a glass rod after introduction of 60 ml of beef extract broth (0.3 per cent). The resulting dense suspension of cells was further diluted with beef extract broth to approximately the same turbidity (Lumetron Photoelectric Colorimeter) as the 0.3-per-cent beef extract broth culture of the corresponding age. Then, each culture, liquid or solid surface growth, was atomized in 0.3-per-cent beef extract broth for aerial dispersion. All cultures were incubated at 30° C.

Settling plate samples were taken under dynamic conditions of chamber operation with Bunting's agar medium for demonstration of variant types in order to determine whether or not there is a selective death of the variants in the airborne state. Standard conditions of chamber atmosphere were held for all determinations. The results obtained are tabulated in Table VI-8. The results of aerial viability determinations shown in this table are to be considered with some reservation because of the results given elsewhere in this report showing the effect of culture resuspension in fresh beef extract broth (Section III of this report). The nutrient agar culture was thusly suspended in beef extract broth for atomization, whereas the beef extract broth culture was atomized directly.

TABLE VI-8

EFFECT OF CULTURE SOURCE ON AERIAL VIABILITY AND DIFFERENTIAL VIABILITY OF CHROMOGENIC VARIANTS EXPOSED TO 65 PER CENT RELATIVE HUMIDITY AT 68°F

Culture	k		BR-%		R-%		P-%		W-%	
	Dynamic	Static	IB†	DYNAM††	IB	DYNAM	IB	DYNAM	IB	DYNAM
2 Day Slant	0.03	0.015	23.9	26.3	69.3	64.3	6.8	8.4	0.0	1.0
2 Day Broth	0.055	0.033	66.3	73.0	30.3	23	2.4	3.0	1.0	1.0
4 Day Slant	0.037	0.018	28.0	29.7	65	59	6.0	11.3	1.0	0.0
4 Day Broth	0.028	0.046	88.0	90	10.0	9.0	1.0	1.0	1.0	0.0
8 Day Slant	0.066	0.029	25.0	30	65	63	9.0	7.0	1.0	0.0
8 Day Broth	0.043	0.042	94.0	92.3	5.0	7.7	1.0	0.0	0.0	0.0
10 Day Slant	0.039	0.021	28	31.8	60	55.2	11	12.0	1.0	1.0
10 Day Broth	0.031	0.045	85	84	12.0	13.0	3.0	2.0	0.0	1.0

†Initial blank; the original inoculum for the cultures was: BR-26.0%, R-64%, P-9.0%, W-1%

††Dynamic settling plates.

A somewhat similar experiment was made toward an investigation of the possible differential viability response among the chromogenic variants. In this procedure, two 60-ml cultures, one of 0.3-per-cent beef extract broth and the other Bunting's medium (liquid), were inoculated with a reconstituted culture to provide the original distribution of chromogenic variants, viz., BR 17 per cent, R 59 per cent, P 22 per cent, W 2 per cent. This reconstitution was effected by a mixing of 1-BR, 6-R, 3-P, and 0-W variant type colonies from Bunting's agar medium. A "bead" of this mixture approximately 1.5 mm in diameter

was diluted to 10^4 in sterile deionized water. Two drops of this suspension were instilled into each of the above media. Following incubation of about 48 hours at 30° C, the cultures were filtered through millipore filters and the filtered cells resuspended in fresh 0.3-per-cent beef extract broth (60 ml) for atomization under the standard test aerial conditions (68° F, 65-per-cent relative humidity). The settling sample plates were Bunting's agar medium for differentiation of chromogenic variants. The results of this experiment are shown in Table VI-9.

TABLE VI-9

CULTURE MEDIA, AERIAL VIABILITY AND DIFFERENTIAL RESPONSE OF CHROMOGENIC VARIANTS

	Aerial Study		Variants - Per Cent							
			BR		R		P		W	
Culture	Conditions	k	IB†	Chamber††	IB	Chamber	IB	Chamber	IB	Chamber
Beef Broth	Dynamic	0.028	64.4	56.3	32.2	39.2	3.4	4.3	0	1
Bunting Medium	Dynamic	0.043	24.2	21.0	57.8	63.0	18.0	14.0	1	2.0
Beef Broth	Static	0.019	52.0	64.2	41.8	31.6	6.2	4.2	0	0
Bunting Medium	Static	0.012	20.1	21.8	63.3	63.6	15.2	12.3	1.4	2.3

†Initial blank. The original inoculum for the various cultures showed: BR 17.5 per cent; R 63.8 per cent; P 18.7 per cent; W 0.0 per cent.

††On settling plates, either dynamic or static.

VII.

CULTURAL CHARACTERISTICS OF *Azotobacter agile*

In the search for methods to measure the water content of airborne bacterial cells it was suggested that a relatively large bacterial cell would be advantageous in the determination of water uptake by cells as indicated by changes in size. Thus, if the mechanical die-away were great enough to be comparable to the biological die-away, it would be possible to make accurate determinations of the average particle size of the viable components of an airborne population at various relative humidities. To meet these requirements the bacterial cell would need to have an equivalent Stoke's diameter of at least 3 microns and preferably 5 microns.

A search of the literature revealed that a species of *Sarcina* was the only vegetative bacteria previously used in aerobiological studies which possessed a cell as large as desired. The difficulty of separating sarcina into individual cells, however, makes this species of little value in aerobiological studies. A search was instituted for a suitable bacterial species which had not previously been used in aerobiological studies.

In addition to the size requirements, a suitable organism would need to be spherical, or nearly so, aerobic, preferably nonsporulating or encysting, nonpathogenic, nonclumping, hardy, nonfastidious as to cultural requirements, and capable of being maintained as a homogeneous culture over long periods of time. Disregarding those intermediate forms which have cell walls probably not typically bacterial in nature, only *Sarcina* and *Azotobacter* appeared to offer any promise of meeting the established requirements.

Further investigation of Azotobacter indicated that Azotobacter agile should meet all the requirements for aerobiological studies, except that of maintenance of homogeneous cultures over long periods of time, extreme pleomorphism being characteristic of all Azotobacter[1]. However, Gunter and Kohn[2] reported some success with A. agile as a test organism. Their work indicated homogeneity of size and shape in their cultures. Accordingly, correspondence was carried out with Dr. Gunter and details of her methods obtained. Similarly, information was sought from Dr. Perry W. Wilson because of his extensive interest in Azotobacter. Cultures were obtained from both Dr. Wilson and Dr. Gunter. Both cultures were labelled "4.4" and appeared to be the same organism, probably Strain 4.4 of C. B. van Niel.

The culture from Wilson contained mostly long rods while that from Gunter contained mostly ellipsoidal forms. After several transfers through Gunter-Kohn liquid[3] (salts plus glucose with no organic nitrogen) the cultures showed more similarity, with a preponderance of elliptical cells. The Wilson culture was discarded on the assumption that it was identical with the Gunter culture. Using culture liquids with no organic source of nitrogen, cell concentrations of greater than 1×10^6 per ml were not obtained, and the more often the culture was transferred through these liquids, the less homogeneous it appeared.

Further considerations of the cultural requirements of A. agile indicated that although a nitrogen fixer, there was no reason to suppose that it could

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[1] H. L. Jensen, "The Azotobacteriaceae," Bacteriological Reviews 18, 195-214 (1954).

[2] S. E. Gunter and H. I. Kohn, "Effect of X-Rays on the Survival of Bacteria and Yeast," Journal of Bacteriology 72, 422-428 (1956).

[3] Gunter and Kohn, Loc. cit.

not effectively handle organic nitrogen. Accordingly, a variety of culture fluids were prepared, inoculated from the Gunter-Kohn cultures, incubated at 25° and 30° C, aerated or shaken, or held still. Twelve culture formulations were employed, varying from plain salts, with or without glucose, with or without fixed nitrogen, with or without peptones or beef extract or yeast extract, with or without Tween-80, and with or without carbonates. These cultures were transferred serially every 48 hours and examined microscopically for (1) total cell counts, (2) cell size and shape, and (3) presence or absence of clumping.

After following the course of the organism through the various cultures for five transfers it became evident that among the culture fluids employed, 0.3-per-cent beef extract broth with 1.0-per-cent glucose on the shaker gave the most consistent and rapid growth of most nearly uniform cells. Applying this information to solid media, it was found that nutrient agar with 1.0-per-cent glucose gave excellent results.

On the basis of the above studies this culture of A. agile is now being carried in 0.3-per-cent beef extract broth with 1.0-per-cent glucose in shaker culture, with plating out on nutrient agar containing 1.0-per-cent glucose. These cultures have maintained their integrity over several weeks.

The cells of A. agile in the uncontaminated cultures are uniformly ellipsoidal, measuring approximately 3 x 4 microns. Preliminary tests indicate that the organism will survive aerial dispersion. Thorough studies, however, will necessitate changes in the present equipment because the prechamber was designed to eliminate particles as large as 3 microns in diameter. Work is

now in progress to design and fabricate a prechamber which will eliminate large particles which might contain more than one bacterial cell, but which will allow the passage of the particles carrying single cells of A. agile. Once this is accomplished, it is hoped that detailed studies will yield information as to whether or not the moisture content of airborne living vegetative cells is dependent on the relative humidity of the air. In these studies, the washed cells of A. agile will be dispersed into the chamber, the concentration of viables in the air determined, and also the numbers of viables settling per unit area per unit time. From these data, taken under various conditions of relative humidity, it will be possible to determine the average size of the viable cells with considerable accuracy, providing the biological die-away is not excessive. The cells of A. agile are sufficiently large that a change in moisture content from approximately 75 per cent to 10 per cent (that concentration of water found in nonviable vegetative cells at very low humidities) will represent a detectable change in size. Such favorable circumstances do not exist for the smaller cells such as those of S. marcescens which have been employed in most of our previous work.

VIII.

STUDIES INITIATED

1. Effect of chemical vapors on washed cells. As an estimate of the susceptibility of washed cells to the aerial environment (see Sections III and IV of this report), several determinations of the effect of aerial disinfectants were made with washed cells. At 80-per-cent relative humidity, employing saturation concentrations, triethylene glycol (hygroscopic) and 2-ethylhexanediol-1,3 (non-hygroscopic) were employed. Previous determinations with cells dispersed from beef extract cultures yielded k values of 0.01 for triethylene glycol, and 0.30 for 2-ethylhexanediol-1,3. For washed cells the corresponding values of k were found to be 0.21 and 0.22, respectively. These findings indicate that the washed cells are in intimate contact with the aerial environment, and are quite capable of serving as condensation surfaces for vapors. The significance of the increased toxicity of the hygroscopic agent is not yet apparent; further investigation will be carried out if possible. In view of the relationship between increased k and changes in growth response (Section IV of this report), similar studies are planned on cells exposed to aerial disinfectants.

2. In vitro toxicity of high concentrations of materials. The correlation of aerial death rates and *in vitro* effects is a primary objective of the present study. However, the difficulty of adequately mixing and sampling high concentration mixtures of beef extract and bacterial suspensions has not been overcome. Present efforts are directed toward a semimicro method. Results with intermediate concentrations of beef extract broth (up to 20 per cent) indicate little or no harmful effect on bacterial suspensions. Work with high concentrations of liquids has been more successful. Reasonable correlation, except

in the case of glycerol, has been obtained between *in vitro* and aerial death rates. With glycerol, aerial *k* at 40-per-cent relative humidity and 68° F is 0.025 (not greatly different from *k* in absence of glycerol), and the *in vitro* *k* at equivalent concentration (86-per-cent glycerol) is very small. At high humidities, however, the aerial death rate is appreciably greater than that incurred in the absence of glycerol. This would suggest that lower concentrations of glycerol are more toxic than higher, a suggestion not borne out by *in vitro* studies.

3. Sampler efficiencies. In connection with the problems involved in sampling bacterial aerosols studies have been directed toward an absolute evaluation of sampler efficiency as contrasted to sampler effectiveness, the latter term including efficiency and also demonstration of viables. For liquid samplers it was decided to evaluate in terms of total particles collected in connection with studies on growth responses (see Section IV of this report). Preliminary work with this technique has been promising and it is hoped that a definite value for the efficiency of the liquid samplers will be forthcoming. Establishment of this value will permit the comparison of other samplers. In the meantime, a procedure has been established for estimating the efficiency of solid surface samplers such as the sieve; comparison of this efficiency with sampler effectiveness in demonstrating viables is expected to be of value. In this procedure, sieve samplers are connected in series, and air samples taken in parallel between the sieves, using 1.0 L/min critical orifice liquid impingers. Assuming that the liquid samplers will operate at the same effectiveness, knowledge of the concentration of airborne organisms entering the first sieve and

the concentration exiting from each sieve thereafter will enable the estimation of the efficiency of the sieve in removing particles. For a concentration of about 3,000/L, the efficiency of individual sieves (six in series) is each approximately 75 per cent at a particle size of 2.0 microns diameter. The effectiveness of the sieve in demonstrating viables is only about 35 per cent, indicating that about half of those viables intercepted are not demonstrated. Because of the length of time which was employed in taking samples with the sieve (5 minutes), it is doubtful that the surface of the agar became dry during the sampling (see Section V of this report).

Although work on samplers is being carried out under a separate project (E-1938), some studies were made under the present project prior to the activation of E-1938. The most significant of these was a comparison of the effectiveness of various fluids in the 1.0 L/min critical orifice liquid impinger with the same fluids in the current Detrick all-glass impinger (DWG-C-93-1-3958). The fluids were brain-heart enriched medium, 0.85-per-cent sodium chloride, 0.2-per-cent gelatin, and deionized water. Relative recoveries with the 1.0 L/min sampler were 100, 70, 60, and 48 per cent respectively. On the same basis, relative recoveries with the Detrick all-glass impinger were 60, 25, 13, and 15 per cent respectively. These data verify the previous contention that the orifice size (sampling rate), geometry and volume of the liquid impingement bed play an important part in the effectiveness of the sampler when handling biological aerosols. (Appl. Microbiol. 5, 119, 1957)

4. Cytological studies on Azotobacter agile. During the course of cultural studies on Azotobacter agile, it was noted that the cells vary in appearance

from dense to reticular. Cells in actively growing cultures most often appear to contain a reticulum of formed material when observed with phase contrast optics. Such cells placed in solutions of AV (bovine albumin, fraction V) for the determination of the refractive index, exhibited phase contrast reversal, but the formed bodies did not, indicating a much lower water content for the formed bodies. In preparations containing a concentration of AV of refractive index equal to that of the cell sap, the details of the formed bodies were easily observed. This technique is to be recommended in the cytological study of A. agile. A. agile will not reproduce in plain AV solutions; the addition of glucose will promote division for cytological studies.

Electron micrographs of cells of A. agile show them to possess many very long flagella, which presumably accounts for their extremely active motility. The cells are too thick for electron micrographs thus far obtained to yield much information about the structure of the formed bodies within the cells.

Washed cells of A. agile are easily ruptured in an ultrasonic field, and a suspension of the formed bodies thus liberated was examined in a solution of AV. It was found that phase contrast reversal was exhibited in solutions of AV of much lower concentration than when these bodies were observed within the intact cells. Whether this means that the formed bodies increased their water content when removed from the cell, or whether it signifies only that phase contrast reversal is a function of the difference between the refractive indices of the two materials in close contact (that is, when within the cell, all that would be determined is the fact that the refractive index of the formed bodies is greater than that of the cell sap) is not known. If the

formed bodies change in water content following removal from the cell, such studies might yield information concerning the exact composition of the cell sap. Thus, a solution of the proper osmotic effect to cause the liberated formed bodies to assume the same refractive index as they exhibit within the cell should give information as to the osmotic differential existing between the formed bodies and the cell sap. Because of the possible application of such data to investigations on the water concentration of living bacterial cells, these studies are being continued.

5. Aerial death rate of washed cells of a microorganism isolated from the laboratory air. In searching for a bacterium which might exhibit a very low death rate, the various organisms which settle out of the laboratory air were examined. It was noted that agar residues left in 2-liter flasks overnight showed many more colonies than did petri dishes of agar exposed for the same length of time. Visual characteristics of such colonies were noted, and among the developed colonies a yellow pigment forming coccoid organism was observed to be invariably present. Almost all other colonies were those of Serratia marcescens or of molds. The regular incidence of these yellow colonies suggested that the organism was well adapted to the aerial environment, and isolates were secured and cultured (0.3-per-cent beef extract broth or nutrient agar, 30° C). Surface growth from nutrient agar was collected, and the cells were washed and suspended in sterile deionized water. Aerial death rates (k) for the washed cells were determined in the aerosol cylinders as 0.029 at 68° F and 44-per-cent relative humidity, 0.021 at 71-per-cent relative humidity, and 0.007 at 95-per-cent relative humidity. Although the values of

k at 44- and 95-per-cent relative humidity are significantly lower than those for washed cells of S. marcescens under these same conditions, these values (except possibly at 95-per-cent relative humidity) are too great to permit the accurate estimation of possible changes in size in response to relative humidity, for such a small microorganism.

6. Comparative viability of bacterial genera. In studying the effect of particle substrate and various atmospheric conditions on the viability of the airborne bacterial cell, it is of value to have a group of bacterial cultures exhibiting a wide divergence of aerial viability. The function of such organisms having either a divergent biological k or fall-out k in such studies is discussed in Section II of this report.

Bacterial organisms having equivalent Stokes diameters of less than 1.5 microns and exhibiting divergent death rate values in response to the atmospheric conditions of 68° F and 65-per-cent relative humidity (standard conditions for culture monitoring) fall into generic groupings relatable to k. Generally, the genus Escherichia exhibits the least viability ($k = 0.09$), the various species of Serratia filling an intermediate position ($k = 0.035$ to 0.05), and the genus Micrococcus showing the greatest resistance ($k = 0.02$) to the airborne state. Some species of Escherichia indicate a degree of variability, particularly in the results from nutrient agar settling plates. The variations, however, occur in the range of values of k 0.06 to 0.09. Cultures of these organisms will be employed in in vitro studies with high concentrations of beef extract broth, to aid in relating in vitro and aerial death rates.

7. Comparison of aerial and in vitro death rates in terms of water content. The generally observed fact that airborne bacteria exhibit a greater death rate at intermediate humidities has elicited a number of suggested explanations, none of which have been verified. In a recent paper, Monk and McCaffrey (J. Bacteriol. 73, 85, 1957) presented data for the in vitro death rates of washed cells of Serratia marcescens in terms of various concentrations of water. From these data these authors suggest that a critical moisture content of approximately 33 per cent is associated with maximum lethality. In an earlier paper dealing with in vitro effect of water concentration in various substrates, Monk, et al. (J. Bacteriol. 72, 368, 1956) had pointed out that this critical moisture content could bear no relation to the aerial situation because the moisture content of washed cells at intermediate relative humidities in the air would be only about 8 per cent (Based on Stevens' data; C. L. Stevens, unpublished data).

As indicated in Section II of this report, we are not convinced that the equilibrium moisture content of dead cells (as studied by Stevens) is necessarily the same as that of living cells under the same conditions. The data compiled in Table VIII-1 offer some corroborations, assuming that the concept of a critical moisture content for maximum lethality is a valid one. In this table the maximal death rates are underscored, and occur under conditions of 33-per-cent moisture content for beef extract solids (airborne particles aerolized from 0.3-per-cent beef extract broth) and for Monk and McCaffrey's washed cells (in vitro). This fact could be interpreted as meaning that the moisture content of the living bacterial cells is more nearly comparable to that of

beef extract solids than it is to the values given by Stevens for dead cells. The data for the death rates of airborne washed cells would then be interpretable in terms of the critical moisture content - maximum lethality concept. Detailed studies on the response of airborne cells to relative humidity, employing dispersing agents which have equilibrium moisture responses radically different from beef extract, will be required before this interpretation can be evaluated.

TABLE VIII-1

COMPARISON OF AERIAL AND IN VITRO DEATH RATES OF CELLS OF *Serratia marcescens* IN TERMS OF EQUILIBRIUM MOISTURE CONTENT

Relative Humidity (%)	Equilibrium Moisture Content of Beef Extract (%)	Aerial k, Cells Surrounded by Beef Extract	Aerial k, Washed Cells	Monk and McCaffrey, in vitro, Washed Cells		Stevens, Equilibration Moisture Content of Washed Cells	
				Water Content (%)	k	R.H. (%)	Water Content (%)
20	10	0.02	0.006	10	0.002	20	6
40	18	0.02	0.006	18	0.01	40	9
60	33	0.03	0.013	33	0.07	60	13
80	46	0.008	0.003	46	0.03	80	21
90	62	0.008	0.002	62	0.01	90	29

8. The effect of a sudden change in relative humidity on airborne bacteria. Data previously reported by this group (Final Report, Project No. B-106) had indicated that the total number of bacteria surviving atomization and equilibration to various lower humidities was inversely related to the final relative

humidity. In Section I of the present report information is given concerning the rates of dehydration of beef extract solids, which make up the greater portion of these bacterial aerosols. This information shows that the rate of diffusion of moisture within the particle is constant; the greater the amount of moisture lost by a particle, the greater the time required for dehydration. Thus, it might be suggested that the previously reported data indicate that the greater the length of time required for drying, the greater the chance of survival of the bacteria within the particles. This explanation lacks merit, and automatically eliminates the concept of a critical moisture content at maximum lethality. Because of this conflict, equipment is being planned suitable for the more exact determination of the total numbers of airborne bacteria surviving under conditions of sudden changes in relative humidity. The equipment previously employed had a detention time of approximately one minute, and considering the equilibration time of the particles involved (less than one second), this may have been excessive.

IX.

DISCUSSION

The factors which determine the ability of an airborne microorganism to be transferred from a source of infection via the air to a susceptible individual are of vital public health interest. This is especially true because the increasing application of air conditioning places more and more people, summer and winter, in essentially closed environments. In all studies of airborne bacteria the water content of the cell or of the nonliving material associated with it assumes importance. Whatever factor may affect the fate of the cell, its action is modified in some fashion by the water content of the particle. In attempting to elucidate the role of water in determining the fate of airborne bacteria, the interdependence of many biological and physical variables must be considered, in order to prevent confusion in ascribing causes. For this reason, in the studies described in this report a variety of approaches have been used; the significance of these is considered in the following paragraphs.

In the normal course of events the airborne bacterial particle of infectious potential arises from some animal source and is associated with relatively large amounts of nonliving proteinaceous materials. Previous studies have indicated that the fate of a bacterial cell under these conditions is determined by the nature and composition of the nonliving material which makes up its immediate environment. It has been suggested by others that the rate at which any change occurs is also important in determining the fate of the cell. Any response of the cell to the ambient atmosphere is indirect, being a direct response to whatever changes have been caused in the immediate particle

environment by the ambient atmosphere. For this reason, studies on the physical characteristics of proteinaceous materials under varying conditions of temperature and humidity must precede studies on the response of the cell to these conditions. In our investigations we have employed beef extract broth as the dispersion medium in most instances because it very closely resembles saliva in its equilibrium moisture content at various humidities. The measurement and analysis of the rates of dehydration and rehydration of microscopic particles of beef extract described in this report show that water is lost from the atomized droplet very rapidly, leaving a residual particle of beef extract. The loss of moisture from this particle is rapid, but being controlled by the internal diffusion of moisture through the particle is not nearly so rapid as the initial water loss. The time required for moisture equilibration of particles, such as employed in our studies, is less than one second.

The results obtained from measuring the rates of dehydration of microscopic particles of beef extract are of considerable value. First of all, they prove fully the contention that our previous work was carried out with equilibrated particles. More important, these results will be useful in evaluating the concept of critical moisture - maximum lethality, insofar as they will enable us to estimate the water content of the airborne particle at any time, and also to estimate the concentration of gradients existing within the particle during equilibration.

The measurement of the rates of rehydration of microscopic particles of beef extract showed that these particles do not rehydrate completely. This work will have to be extended because of its potential significance in

evaluating rehydration of particles inhaled into the respiratory apparatus of man, and its value in explaining some of the anomalous responses of dust-borne bacteria.

In addition to rate of change of water concentration, the actual water concentration at any time is considered to be of extreme importance in the ultimate fate of the airborne bacterium. Published data on equilibrium moisture contents of bacterial cells have been derived from the study of nonviable, lyophilized cells, and show very low moisture contents for these cells (approximately 8 per cent at intermediate humidities). We have considered the water content of living vegetative bacterial cells as not necessarily identical to that of dead cells, especially lyophilized ones. In comparing our data for aerial viability of bacteria surrounded by beef extract to that reported by others in support of the concept of critical moisture content - maximum lethality, we find that the moisture content of the beef extract at intermediate humidity (maximum lethality) is the same as that reported by these workers to effect maximum lethality in vitro (approximately 33-per-cent water). If the concept has validity, this constitutes inferential proof that living vegetative cells have a much greater moisture content than dead, lyophilized cells.

An applicable method for determining the moisture content of living airborne cells is the estimation of size from aerial concentrations and settling data (in terms of viables). However, the test organisms employed thus far have been too small for this method to distinguish changes in cell size. A much larger organism, which is believed suitable, has been successfully cultured and will be used for this purpose. In the meantime, the method of phase

contrast reversal to determine the refractive index of cellular materials (and thus the protein and water concentration) has been applied successfully to the test organisms. Viability is proven either by reproduction in the suspending medium, or by motility. Work is being carried out employing this method, varying the osmotic pressures to simulate the conditions existing within the airborne bacterial particle at various humidities. Results from such studies, coupled with those from size determinations on larger airborne bacteria, will furnish concrete evidence as to whether or not the living vegetative cell experiences large changes in water concentration under conditions of varying relative humidity.

In studying the effects upon the bacterial cell of changes in the immediate environment, the aerial state offers some promise as a method of investigating some of these effects. Thus, the study of cells dispersed from pure water should yield information of value in distinguishing between effects attributable to the immediate environment and those attributable to the aerial state itself. Cells dispersed into the air from pure water have been found to respond in a manner similar to those dispersed from beef broth, but to exhibit generally greater death rates. Exposure to aerial disinfectants of cells dispersed from pure water produces death rates somewhat similar to those exhibited by cells dispersed from beef broth under the same conditions. This is direct evidence that the cell itself acts as a condensation nucleus, and that it remains permeable to the compounds under study. Further investigations of this nature are planned because of the value of the results in describing the permeability of the cell wall to various compounds.

The prior condition of the bacterial cell has often been considered an important factor in aerobiological studies. Studies on manipulation prior to aerial dispersion described in this report have indicated the possible presence of a toxic substance in liquid cultures in the maximum stationary phase of growth. Other results herein reported suggest the presence of an interfering substance produced by one of the variant forms of the test organism. Both of these discoveries may assume more importance in physiological investigations of bacterial cultures than in aerobiological experimentation, but the recognition and avoidance of such problems is of no small value. Studies are being continued on the relation between prior condition and ultimate fate of airborne bacteria.

Although survival or death is the most striking evidence of the response of the airborne bacteria to changes in its immediate environment, numerous attempts have been made to establish criteria for the evaluation of more subtle changes, short of death. The search for these has been given impetus by the consistent finding that enrichment of collection fluids increases the numbers of viables, indicating that a sensitive, or insulted segment of the aerial population does exist. One method of characterizing this sensitive segment of the population has been described in this report--that of determining the growth curves of captured airborne bacteria. It has been found that the length of the initial stationary phase of growth correlates with increasing values of aerial death rate. A more sensitive technique has been developed for this purpose, whereby the total number of cells, the relative number capable of initiating division, the relative number capable of colony formation, and the times of

division are all determined. Currently, this method is being tested under controlled conditions, and will be applied particularly to situations yielding a variety of values of death rates, such as obtained with aerial disinfectants.

Sampling procedures constitute another group of variables which must be considered in aerobiological studies. Our previous experiences with plain fluids in samplers proved the fallacy inherent in using relative recoveries in experimental work. Currently, certain results appear to indicate that even enriched fluids may not demonstrate all the possible viables in an airborne population. This will necessitate further study of these samplers. In the meantime, comparison with other accepted aerobiological samplers has shown that our standard liquid sampler is two to three times more effective in demonstrating viables than any others tested.

The conflicting results between liquid samples and agar samples of high temperature or low humidity led us to believe that the agar surface was at fault. However, a thorough study of the water film at the surface of agar has proven that this film exists for long periods of time in still air, and for many minutes in turbulent air. We must therefore conclude that the source of this problem is not the agar surface, but some characteristic of the airborne particle under these conditions. It is hoped that the completion of the evaluation of the rehydration characteristics of these particles will offer a reasonable explanation of this phenomenon.

Two important approaches to the evaluation of the role of water in determining the fate of airborne bacteria have not as yet been fully exploited because of developmental work required to produce more precise methods. In

order to quantitatively characterize the effect of the high concentration of solids existing in the immediate environment of the airborne microorganism, in vitro studies of similar solids concentrations must be performed. Adequate sampling of mixtures of semisolids is difficult to carry out accurately, and present efforts are directed toward a semimicro method for this purpose. The data presented in this report for the dehydration times of airborne particles are interpreted as signifying that our previous explanation of the effect of sudden changes in relative humidity may be in error. The previous work was carried out in a system having a detention time of one minute; present results show equilibration times to be less than one second. A system with a much shorter detention time is being designed for a more precise study of equilibration effects.

Although a great deal has been accomplished during this first year on the primary objectives of the project, much remains to be done. In addition to the work originally planned, certain findings should be exploited. The unexpected finding that particles of beef broth do not rehydrate to their original water content when cycled from high to low humidities and back has such potential significance in the rehydration of particles in lungs and in the anomalous behaviour of dust bacteria that this work should be greatly expanded. The discovery of possible toxic substances in bacterial cultures through aerial experimentation suggests the value of this approach in physiological investigations, and perhaps offers a new tool in aerial experimentation. The correlation shown between aerial death rate and changes in the growth response of captured airborne bacteria presents direct evidence of effects short of

death occasioned by the aerial state. This finding should be utilized in evaluating the possible relation of the ability of airborne bacteria to successfully act as infectious agents. The response to aerial disinfectants of cells dispersed from pure water suggests the value of this procedure in studying cell permeability to various compounds. It is hoped that current proposals for support of investigations along these lines will be favorably received, permitting the logical development of researches growing out of those currently active.

X.

SIGNIFICANT ACCOMPLISHMENTS

At this stage in the development of the current investigations no particularly significant research accomplishment can be singled out, the investigations having been carried out as a series of parallel studies, none of which are considered to be complete at this time. The significance of these studies is considered in the preceding section (IX) of this report. The overall rate of progress has been good, although some studies have yielded few results because of unexpected diffficulties in the development of methods. On the other hand, the results of certain studies have thrown unexpected light on related problems.

The only publication resulting from the current researches will appear in the 1958 issue of Bacteriological Proceedings ("Correlations Between Aerial Death Rate and Growth Response of Captured Airborne Serratia marcescens," T. W. Kethley, E. L. Fincher and W. B. Cown), and will be presented at the 1958 meeting of the Society of American Bacteriologists. It is worthy of mention that nine publications resulted from the earlier researches carried out by our group under grant-in-aid G-2771. Six of these publications appeared after the completion of that project. It is anticipated that a similar program of publication will be followed for the current project.

Through the efforts of the members of the study group and the Public Health Liaison Officer at Camp Detrick, a most significant conference was held between members of our Aerobiology Group and interested workers of the Army Biological Warfare Laboratories. The subject of the conference was the state of water in bacterial cells. Not only did this conference accomplish an interchange of

information and ideas, but some continuing liaison was established between the two groups. In this connection, it should be pointed out that, through other channels (technical meetings and correspondence) continuing liaison has been established between our group and others concerned with various aspects of aerobiology or directly related fields. To the best of our knowledge these include all of the interested groups in the United States, England and Australia.

XI.

FUTURE WORK

A discussion of the research carried out thus far and the future work indicated to exploit these results is given in Section IX of this report. Only a brief tabulation of future investigations planned is given here; the significance of the work has been considered in the previous section.

Completion of studies outlined in the original proposal.-

1. Detailed studies on the growth curve responses of captured airborne bacteria exposed to various relative humidities, employing solutions of various dispersing agents.

2. Studies on the effect of varying the concentration of solids in the dispersion medium to effect increases in the size of the airborne particles (and thus vary the dehydration time), in terms of k versus relative humidity.

3. Determination at various relative humidities of the size response of washed cells of Azotobacter agile to the airborne state in order to determine the equilibrium moisture content of living cells.

4. Determination of the response of various test organisms to aerial dispersal from various media having equilibrium moisture contents differing from those of beef extract.

5. The exact determination of the effect of sudden changes of relative humidity on airborne bacteria.

6. In vitro determination by a semimicro method, of the effect of high concentrations of materials employed in dispersion media on the various test organisms utilized in aerial studies.

7. The estimation of the effect of osmotic pressures, which simulate aerial conditions, on living cells by phase contrast reversal methods, or preferably by interference microscopy.

New studies originating from the results of current investigations.-

1. Complete investigation of the rehydration of microscopic particles of proteinaceous materials, including preliminary studies on dust-borne bacteria.

2. Application of knowledge of the dehydration process to a semimicro method of studying the effect of high concentrations of solutes on viable bacteria in an attempt to evaluate the effect of rate of dehydration on viability.

3. Investigation of the effect of exposure to aerial disinfectants on the growth response of airborne bacteria.

4. Investigation of the permeability of airborne cells, employing cells dispersed from water and then exposed to aerial disinfectants.

5. Further studies on the absolute efficiency of the 1.0 L/min critical orifice liquid impinger, based on the possibility that even this sampler does not demonstrate all of the viables under certain conditions.

6. Detailed studies on the various factors relating to the effect upon aerial survival of the prior condition of the bacterial cell.

7. Extension of the use of various species and variants within a species as an aid to the evaluation of the effect on airborne bacteria of prior condition and aerial insult.

8. Respiration studies on captured airborne bacteria, in an effort to explain the significance of the relation between aerial death rate and changes in growth response.

9. Investigation of the use of tagged water to determine the water content of living cells.

Compilation, analysis and synthesis of the information obtained from the various approaches in an attempt to present orderly explanations of the role of water in determining the fate of airborne microorganisms.- At this time it is evident that we will not be able to complete, within the current year, the investigations required for the achievement of the primary objectives of this project; certainly, it will be impossible to consummate the investigations which are indicated as logical outgrowths of the researches described in this report. For these reasons, a proposal is being submitted for continuing support of this project for at least another year beyond the current one.

In considering future work by our group on aerobiological problems, attention should be called to the fact that new quarters for this group will probably be available early in 1959. These quarters have been planned especially for our Aerobiology Group, and will represent a considerable increase in useful laboratory facilities. The planned building is a combined radioisotopes and bioengineering facility, partial support for the public health related research portions of the building having been obtained from the Health Research Facilities Branch of the National Institutes of Health. In this building, Bioengineering (consisting of the Aerobiology Group, Radiation Biology Group and Publich Health Research Group) will occupy some 6,000 feet of floor space devoted to offices and laboratories. Of this space, Aerobiology will occupy almost exactly one-half.

The planned facilities for Aerobiology consist primarily of a large workroom having its own temperature and humidity controls, equivalent to those now employed in our present workroom. This increase in size will permit the

carrying out of several investigations simultaneously, and will furnish space for additional experimental chambers. In addition, Aerobiology will occupy an enlarged culture room which will be completely equipped for all the requirements of this group, including the safe handling of pathogenic organisms. The remainder of the space occupied by the Aerobiology Group in the new building will be taken up by autoclave and dishwashing facilities, instrument room, mechanical equipment for the workroom, and offices. This group will also have access to an animal room, 68° F darkroom, herbarium, refrigerator room (containing large reach-in units and a walk-in unit), conference room, and central secretarial service. The close proximity of location to the Radiation Biology and Public Health Groups will greatly enhance the research opportunities both in terms of additional facilites and equipment, and also in terms of personnel for discussion of common problems. Location in the same building with the Radioisotopes Group will make available to the Aerobiology Group the specialized facilities, equipment, and personnel in the fields of radioisotopes and neutron physics.

XII.

APPENDIX

(Reprint of "Equipment" and "Bacteriological Methods and Cultures," from Final Report Project No. B-106.)

III. EQUIPMENT

A. General Description

The temperature-humidity laboratory used to carry out the aerosol work is a heavily insulated room 12 by 30 feet, 6.5 feet high. The room is divided into two sections--the larger serving as the workroom and the smaller serving as the control room for regulating the temperature and humidity. Figure 1 is a photograph showing the interior of the laboratory as observed from the entrance of the workroom looking back into the control room. On the right, the main aerosol chamber, the outer unit of the aerosol cylinders, and the meter chamber are shown. The conditioned-air entrance to the workroom is located over the opening into the control room with the remainder of the opening serving both as an air return and as a passageway between the two rooms. On the left, the instrument for recording the dew-point and dry-bulb temperatures, as well as tables that provide desirable work space for handling plates, samplers, test instruments, etc. are shown. The wet-test meter used in calibrating critical-orifice impingers, nozzles, and flow meters is located on the table in the foreground.

The interior surface of the walls and ceiling of the laboratory is enameled metal. The floor of the workroom is varnished wood, and the floor in the control room is watertight Masonite. These surfaces can be readily cleaned with a good detergent and water. Water, air, steam, vacuum, and electrical services are provided where needed. Also, the construction of the walls is such that openings for conduit and ducts are easily made.

The temperature range for the workroom can be controlled from 0° to 95° F, and the dew point of the air in the room can be controlled from 25° to 95° F,

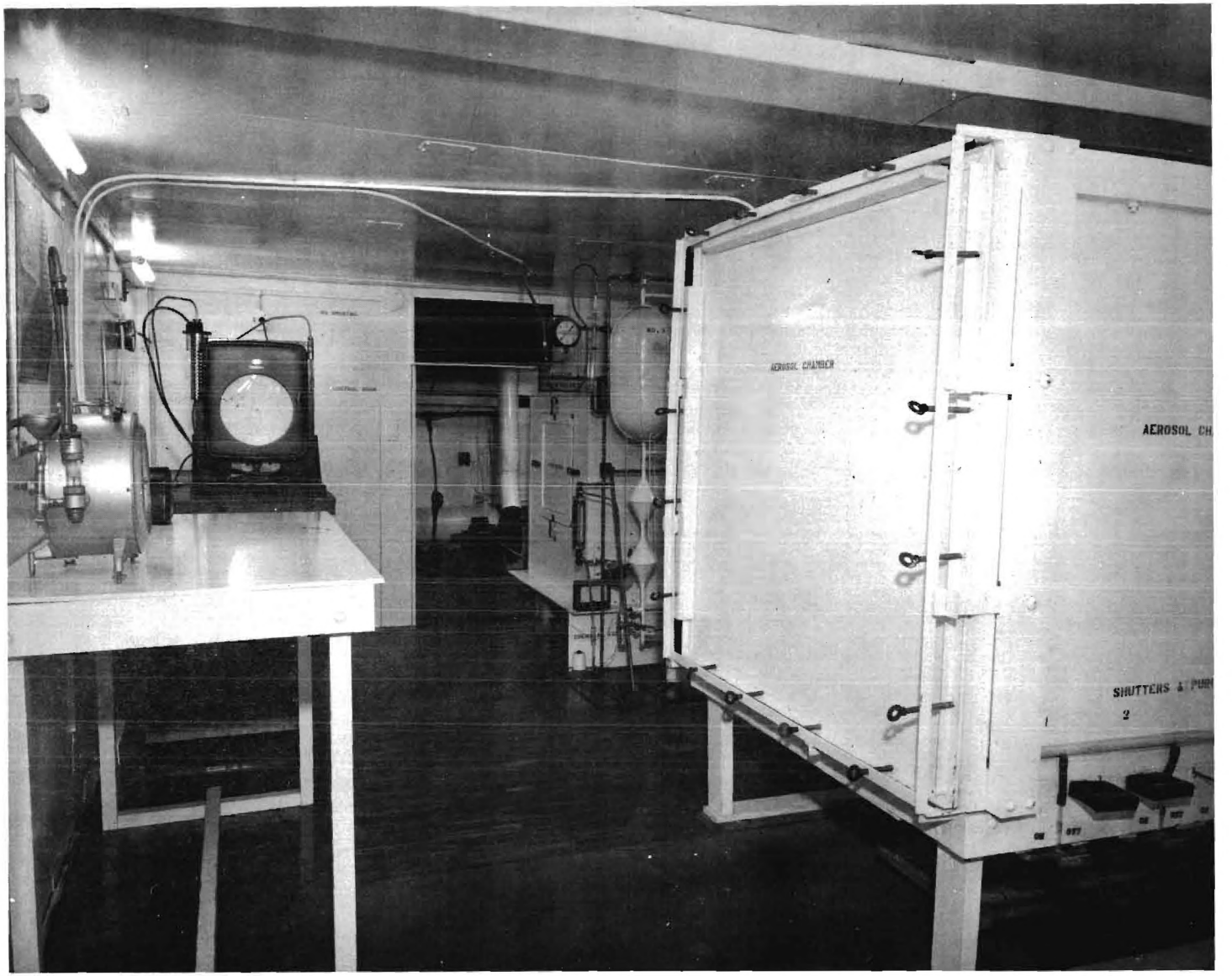


Figure 1. Interior of Workroom.

resulting in a wide range of temperature and humidity (controllable within 1° F for both dry-bulb and dew-point temperatures). The actual conditioning of the air is carried out in the control room. The interior of the control room as viewed from the workroom is shown in Figure 2. The cold evaporators, water reservoir, and pump which circulates the water over the evaporator coils, and the heat exchanger with blower may be readily identified. An inlet for fresh air, though not shown, is located to the right of evaporator No. 2. Also, a steam jet for injecting live steam into the heat blower system for humidification is not discernible. The treated air leaves at the top of the two evaporators through the large ducts along the ceiling which converge just prior to entering the workroom. The blowers in the evaporators combined with the heat blower circulate the air into and from the workroom at about 3,000 cfm. Approximately 60 cfm of fresh, filtered air is continually pumped into the control room through a stack having access to a point outside and above the research building. This air is vented by the aerosol chamber during dynamic runs and through relief shutters or by the meter chamber exhaust system when the chamber pump is not operating. This small amount of makeup air is generally sufficient for comfort because operating personnel in the workroom are kept to a minimum and smoking is prohibited within the room. The desired condition of temperature and humidity is set up by adjusting the temperature of the circulating water, the wet-bulb thermostat which controls the dampers regulating the per cent of air drawn through the evaporator coils, and the dry-bulb thermostat which controls the flow of steam through the heat-exchanger coils. These controls are only semiautomatic until the set point is achieved; intermittent attention is required during the first hour or so

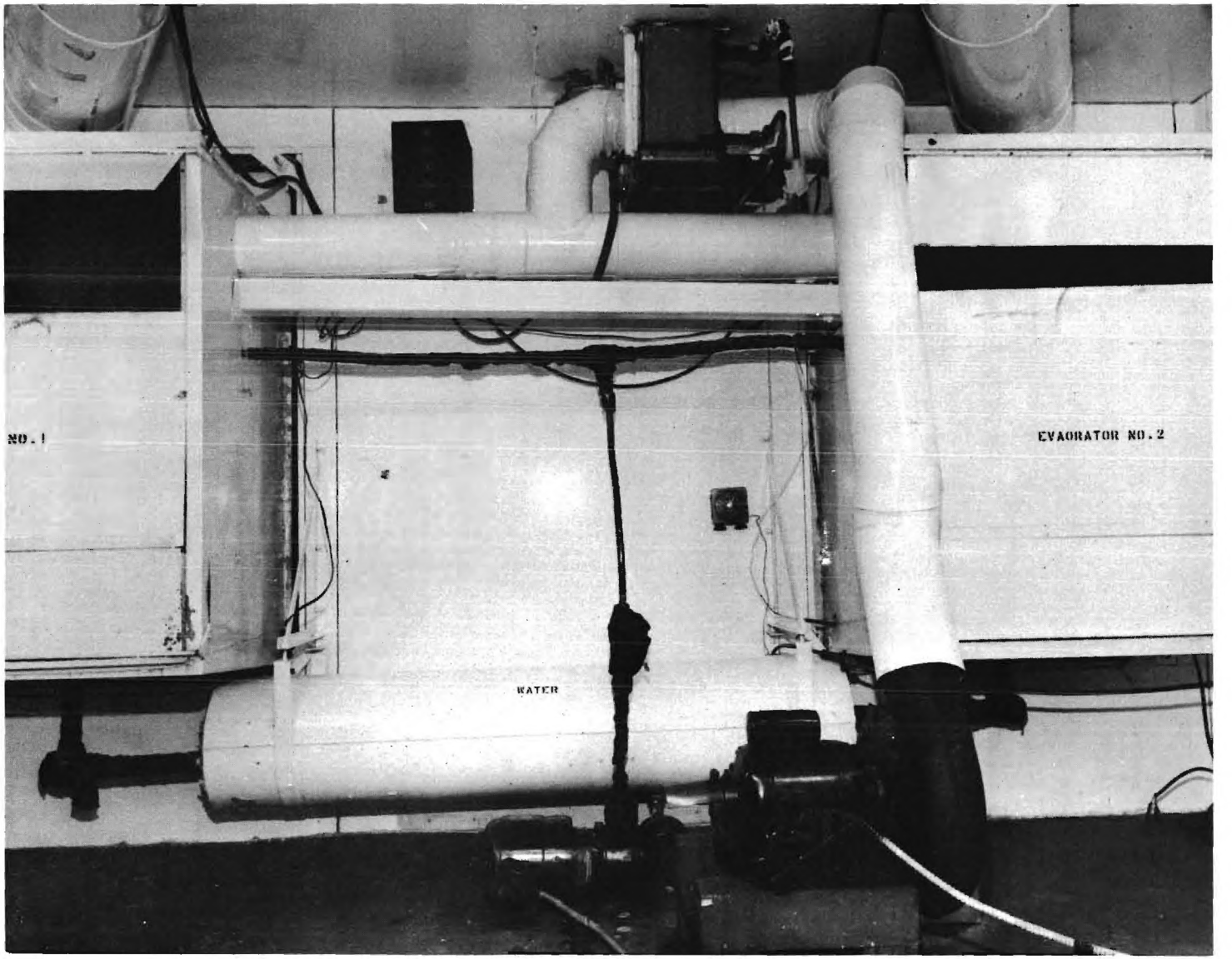


Figure 2. Control Room.

of operation as the controls must be adjusted in relation to the ambient temperature in the building and the temperature and dew point of the outside air. For the higher humidities, live steam is injected at a constant rate; for the lower humidities and temperatures, the fresh-air intake may be closed off.

The aerosol chamber as seen in Figure 1 is located in the right corner of the workroom, about 3 feet from the side walls, and is about 6 inches from the ceiling and 18 inches from the floor. Since the air in the room is circulated at approximately 3,000 cfm, the narrow spacing between the top of the chamber and the ceiling of the room is no deterrent to adequate heat transfer between the room and the chamber.

The chamber itself is a 4-foot cube constructed of tempered Masonite, and one side--the front--is removable. There are no internal projections in the chamber other than the air diffuser. It is 5 inches in diameter and projects from the center of the ceiling into the chamber about 2 inches. The internal surfaces are finished with several coats of a white alkyd resin enamel, each coat hand rubbed to a mirror finish. There are 17 openings in the chamber walls: ten 3/4-inch holes in the left wall for taking air samples; one 3/4-inch hole in the rear wall for connecting a manometer; one 3-inch hole in the center of the ceiling for the air inlet; five 4-inch holes equally spread along a diagonal of the chamber floor, the center one serving as the air outlet, and the remainder are ports for settling samples. The interior of the chamber as viewed with the front side removed is shown in Figure 3. The various sampling ports, outlet hole, and diffuser inlet may be readily identified. Also shown is the frame and screw clamp assembly required for closing tightly the removable side.

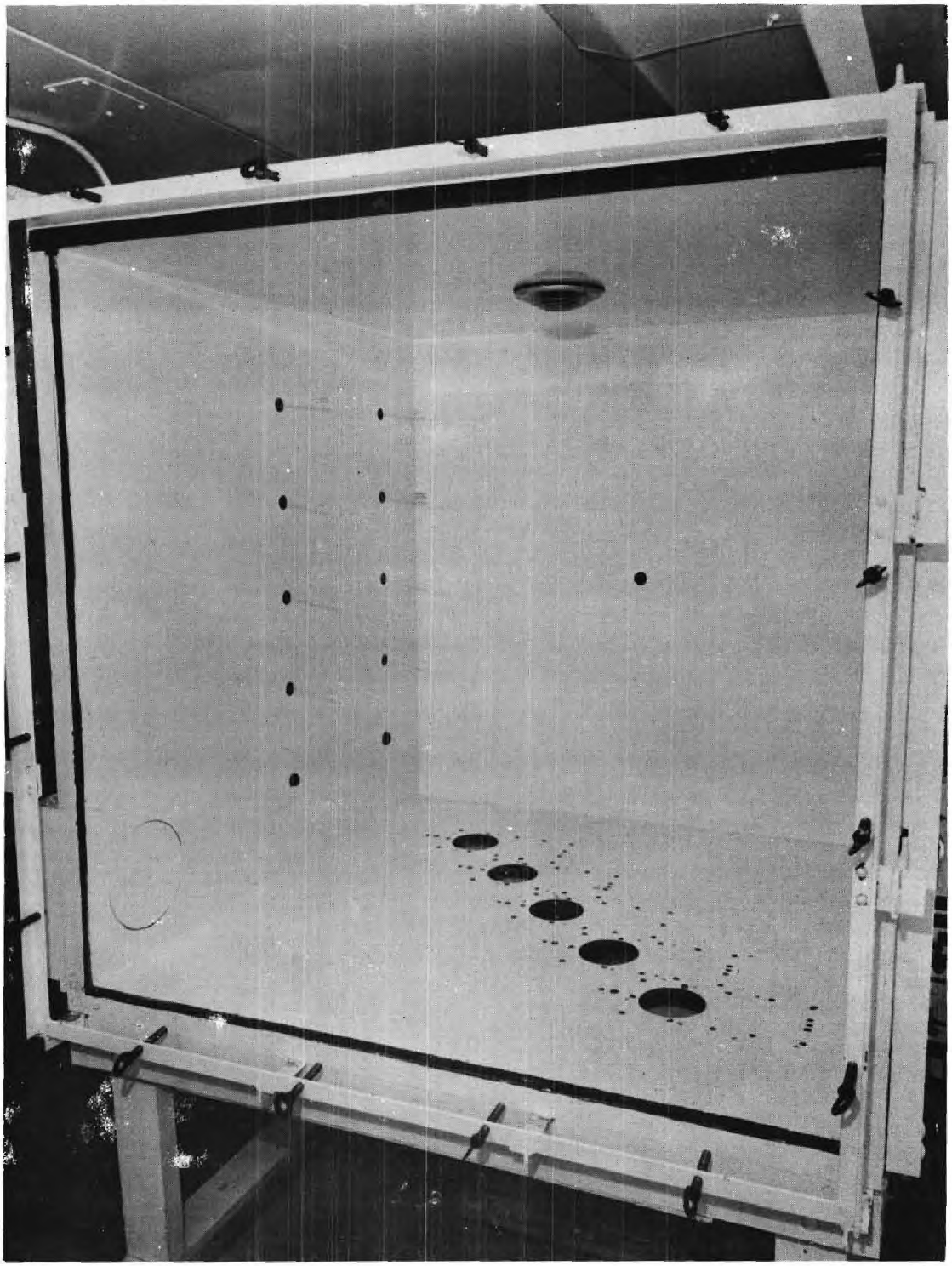


Figure 3. Interior of Aerosol Chamber

A schematic drawing of the chamber and atomizing equipment is shown in Figure 4. During the dynamic runs, the main air stream of the chamber (60 cfm) is drawn from the workroom through filter A and is metered by orifice C, passing through Anemostat D (trade-mark of the Anemostat Corporation of America) and is discharged outside the workroom through filter F. The pressure within the chamber is balanced to that of the surrounding room with the by-pass damper arrangement G, the pressure being read on a manometer.

Clean, regulated, compressed air for the atomizer is metered at I and disperses the bacterial suspension contained in the atomizer J (DeVilbiss No. 40, operated at 6.4 l./min at 20 psi). The resultant aerosol suspension is forced into the 20-inch cubical prechamber K, where the large particles drop out. The air-borne bacteria are thoroughly mixed with the main air stream in the turbulence created at the orifice meter, and then they are uniformly distributed throughout the chamber by the Anemostat. Air samples are then taken from any of the ports indicated for that purpose. Figure 5 shows a number of liquid impinger samplers, which are connected to the air sampling tubes in the side of the chamber and also to the vacuum manifolds. The holders for the samplers slide up and down on the vertical support rods to facilitate the spacing of the samplers and to handle variously sized samplers. (Each individual pump pulls approximately 2 cfm at 23 inches of mercury.) The two vacuum manifolds may be interconnected or used separately with independent vacuum pumps depending on the desired arrangement. Figure 6 is quite similar to Figure 5 but shows the use of heated jackets for controlling sampler temperatures. Although not very discernible in the photograph, these jackets consist of a 6-inch plastic tube with a heating element of resistance wire

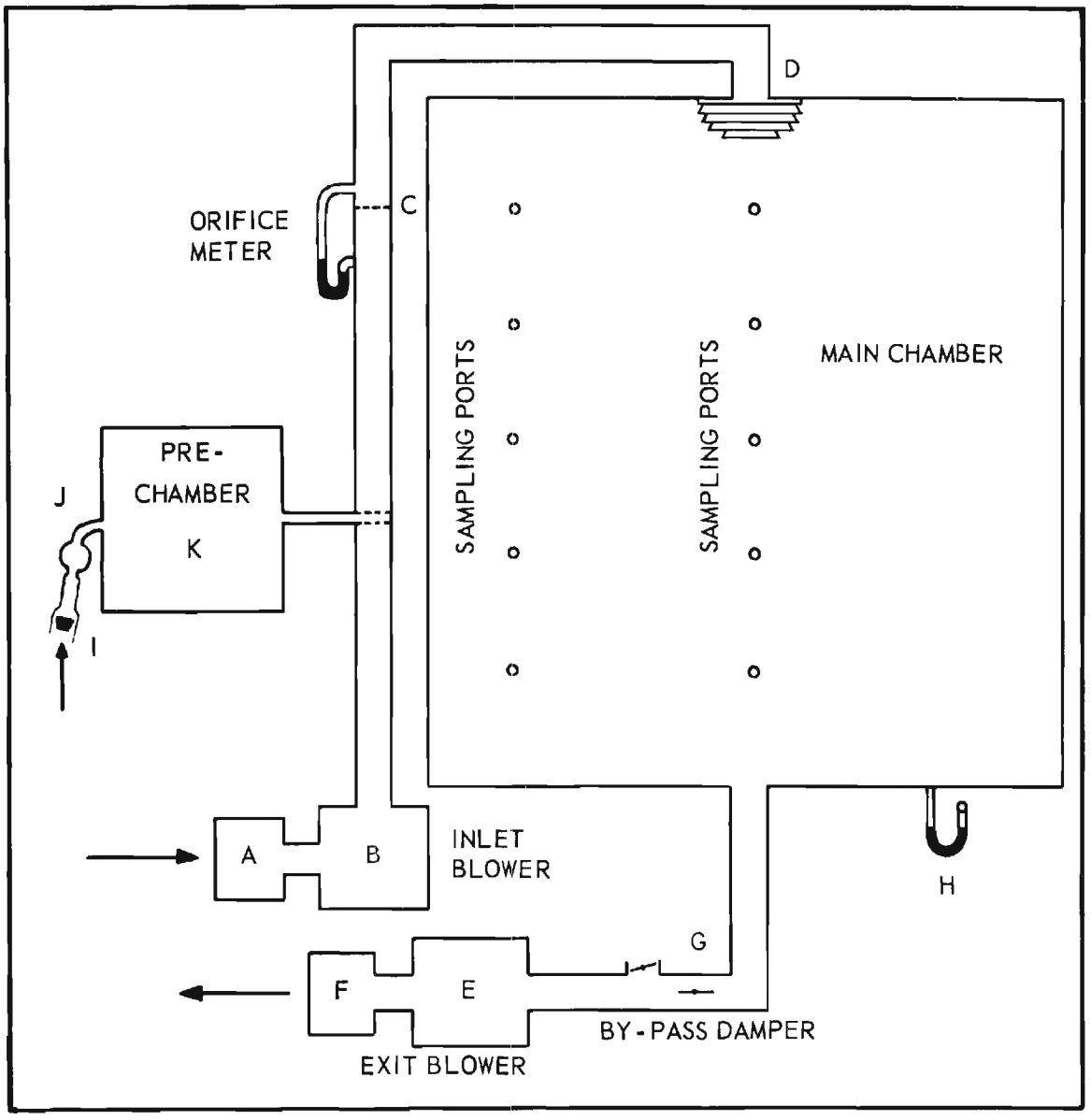


Figure 4. Schematic Drawing of Chamber and Atomizing Equipment.

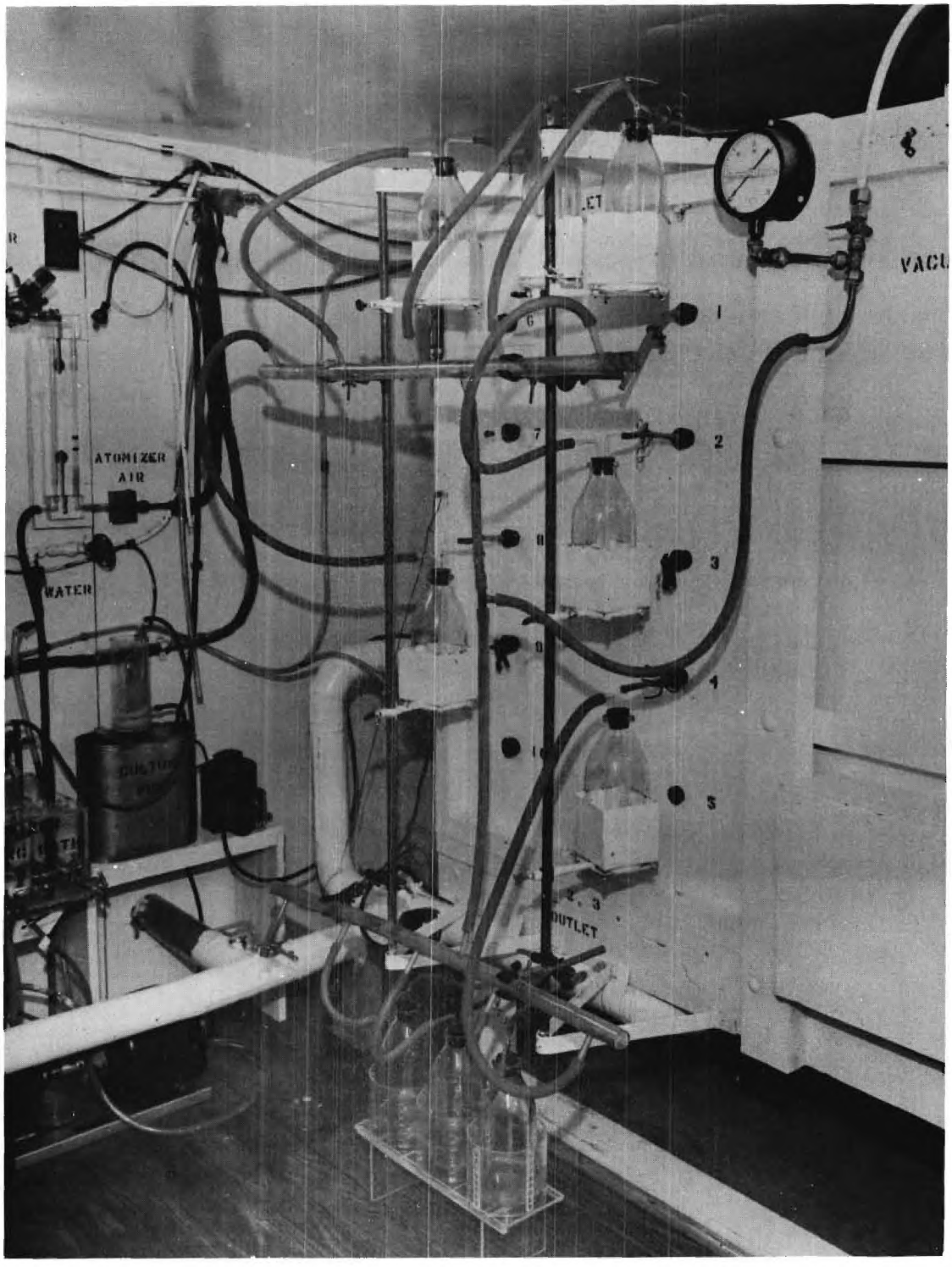


Figure 5. Samplers in Position at Aerosol Chamber.

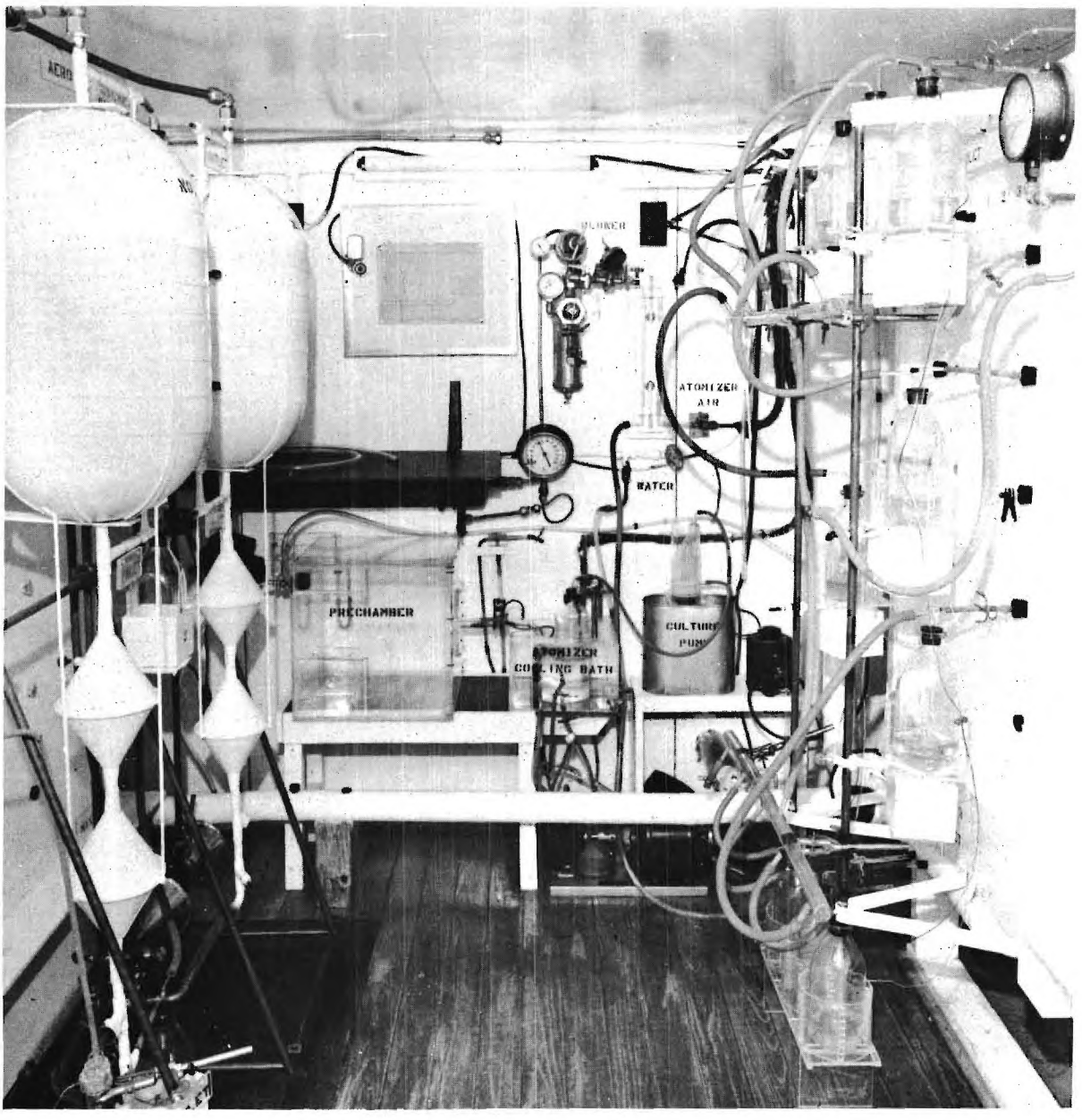


Figure 6. General View of Samplers, Atomizer, and Cylinders.

offset about 1/2 inch within the inner circumference of the tube. Extension tops and felt covers may be added to these jackets when it is necessary to insulate and heat the entire outside surface of the samplers.

The details of the assembly used for air sampling are shown in Figure 7. In use, the milk bottle containing the sampler fluid is sterilized as shown in the center of the center of this photograph. A sterile impinger assembly is inserted just prior to sampling.

The equipment for atomizing the bacterial suspension can be seen in the central background of Figure 6. In order to minimize changes in the bacterial culture during long runs, the atomizer and the 5-inch diameter aspirator bottle used as a reservoir for the culture (see Figure 8) are immersed in a small water bath, the temperature of which is maintained at 45° F. Because the reservoir bottle has a much greater cross-sectional diameter than the atomizer, the level of the culture in the atomizer does not change appreciably for several hours, and it is possible to operate for as long as 10 hours by slightly adjusting the level of the reservoir bottle every 2 hours. Also, to minimize concentration effects within the atomizer, a siphon pump is connected to the atomizer-reservoir system which keeps the culture in the atomizer and that in the reservoir continually mixing with each other. As seen in Figure 6, the atomizing air is metered by the flowmeter just beneath the blower switch before going to the atomizer in the cooling bath. The output of the atomizer flows into the prechamber from which it can then either be directed to a throw-away filter system, to the aerosol chamber on the right, or to the aerosol cylinders on the left, depending upon the situation desired. The regulator to the left of the blower switch controls the air for the settling-plate holder, air-purging

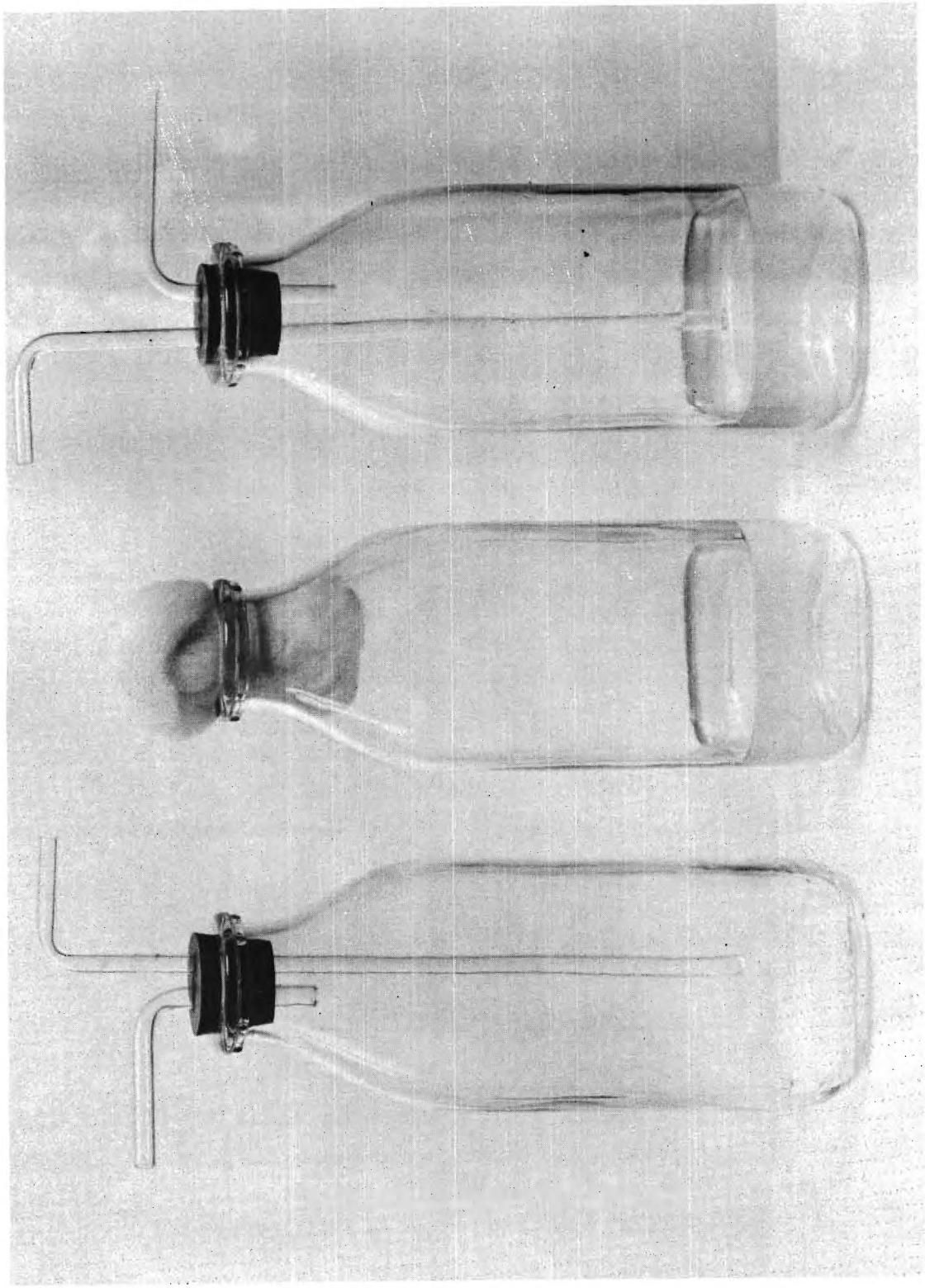


Figure 7. Critical-Orifice Liquid Impingers.

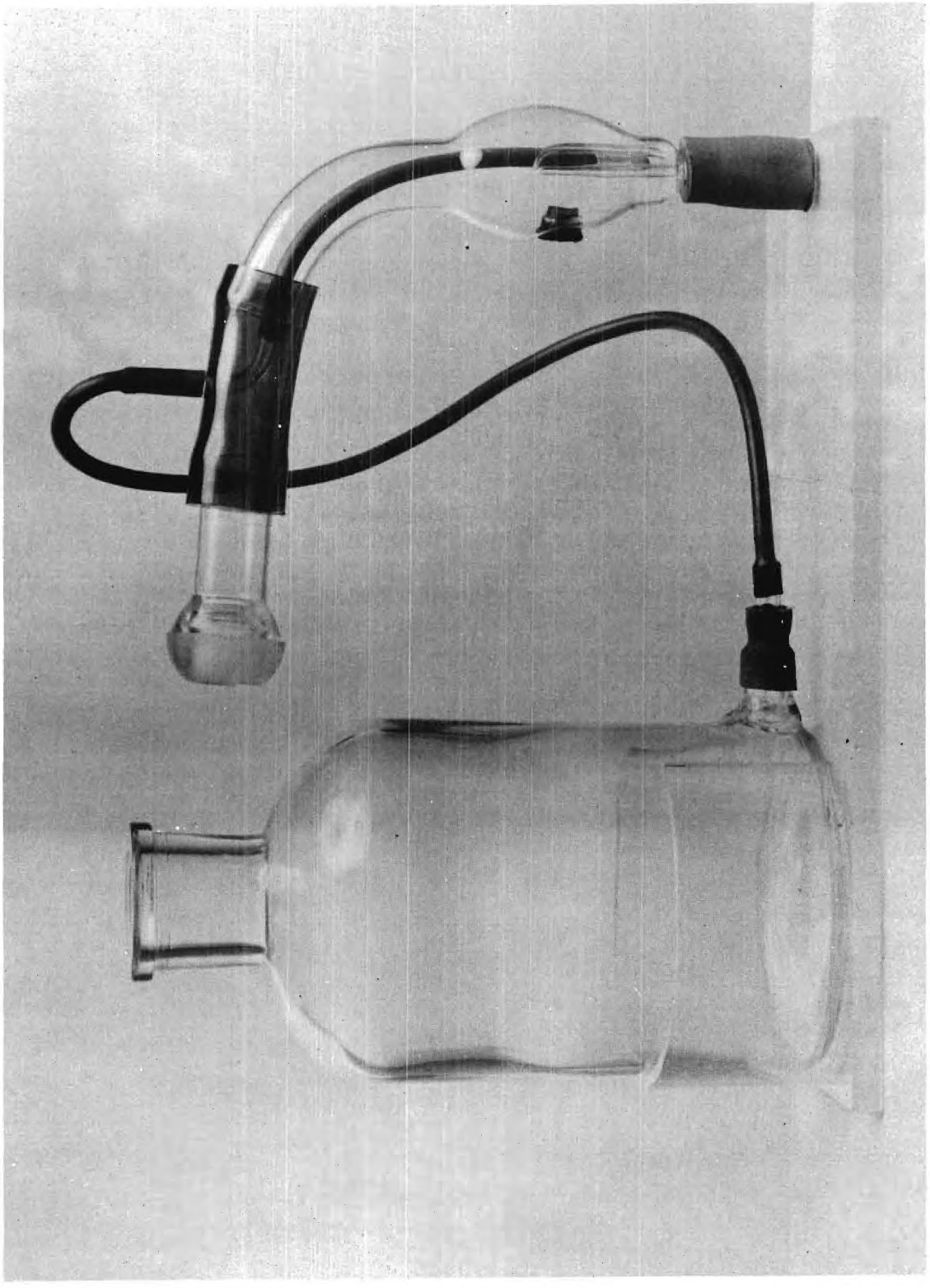


Figure 8. Reservoir and Atomizer.

system, or other uses, and has nothing to do with the atomizer unit. The air supply to the atomizing system is treated rather extensively to insure adequate and uniform pressure, temperature, and humidity regulation. Air from the compressor which furnishes air for general services within the research building is filtered and reduced from approximately 125 to 60 psi with a Binks No. 83 A regulator (Binks Manufacturing Company) and then further reduced and controlled at 30 psi by a Cash special pressure-controlling valve (A. V. Cash Company). Then the air is passed through two oxygen-type cylinders, 35-liter capacity each, located in a refrigerated unit maintained at 40° F. This part of the system serves as a surge tank and regulates, to a certain extent, the humidity of the air. At the exit of the surge tank units, but also in the refrigerated unit to assure constant temperature, is located a more sensitive Cash pressure-regulating valve, which maintains the air at a very constant pressure of 20 psi. It is this air which issues from the valve marked "Atomizer Air" in Figure 6.

The aerosol chamber can be operated either dynamically or statically. In the latter case, the chamber is sealed off by closing the sliding valves located in the entrance and exit pipes of the chamber. Settling samples can be taken within the chamber during each type of operation. The settling sample containers are located beneath each of the 4-inch holes in the bottom of the chamber. The actual sample holders were machined 1-inch diestock. Each holder contains a shutter for sealing off the 4-inch hole in the floor of the chamber, a cavity for holding Petri dishes, and a door on the bottom which can be closed so as to be airtight. In operation all components are airtight under at least 1 inch of water differential, so that when the sliding shutter is closed

the chamber hole is sealed, a Petri dish can be inserted into the cavity of the holder, and the bottom door of the holder closed. Then the Petri dish can be exposed to the chamber by opening the sliding shutter. Thus, it is possible to take settling samples as desired without disturbing the conditions within the chamber. Each settling sample holder is equipped with a small air inlet and outlet, controlled by a three-way stopcock, in order to purge the cavity of the holder after the Petri dish has been exposed. The control handles which operate the settling port slide covers and the valve to the chamber exit are shown in Figure 9 with the center pull being that for the exit valve. The stopcocks for operating the purging system for the settling-plate holder cavities may be seen just below the slide pulls, and the manometer indicating proper balance of inlet-exit flow of the purge system is located on the projection near the top of the chamber side. In the background and to the right of the chamber is the filter and exhaust blower, or exit. The three sampling tubes in the duct leading from the exit blower to the outside of the workroom are used for checking the efficiency and condition of the filter unit.

The inlet blower for the chamber draws air from the workroom or from a cubic-meter chamber which is employed for setting up concentrations of chemical vapors to be studied in the chamber. The exit blower for the chamber discharges through a filter system and outside the workroom.

The aerosol cylinder system and chemical-vapor-generating unit is shown in detail in Figure 10. Flexible stand construction was used to allow for later changes and addition of a third cylinder, if desired. Though aerosol is run through only one cylinder unit at a time, two basic cylinder units are arranged so that the aerosol flow may be directed immediately from one to the

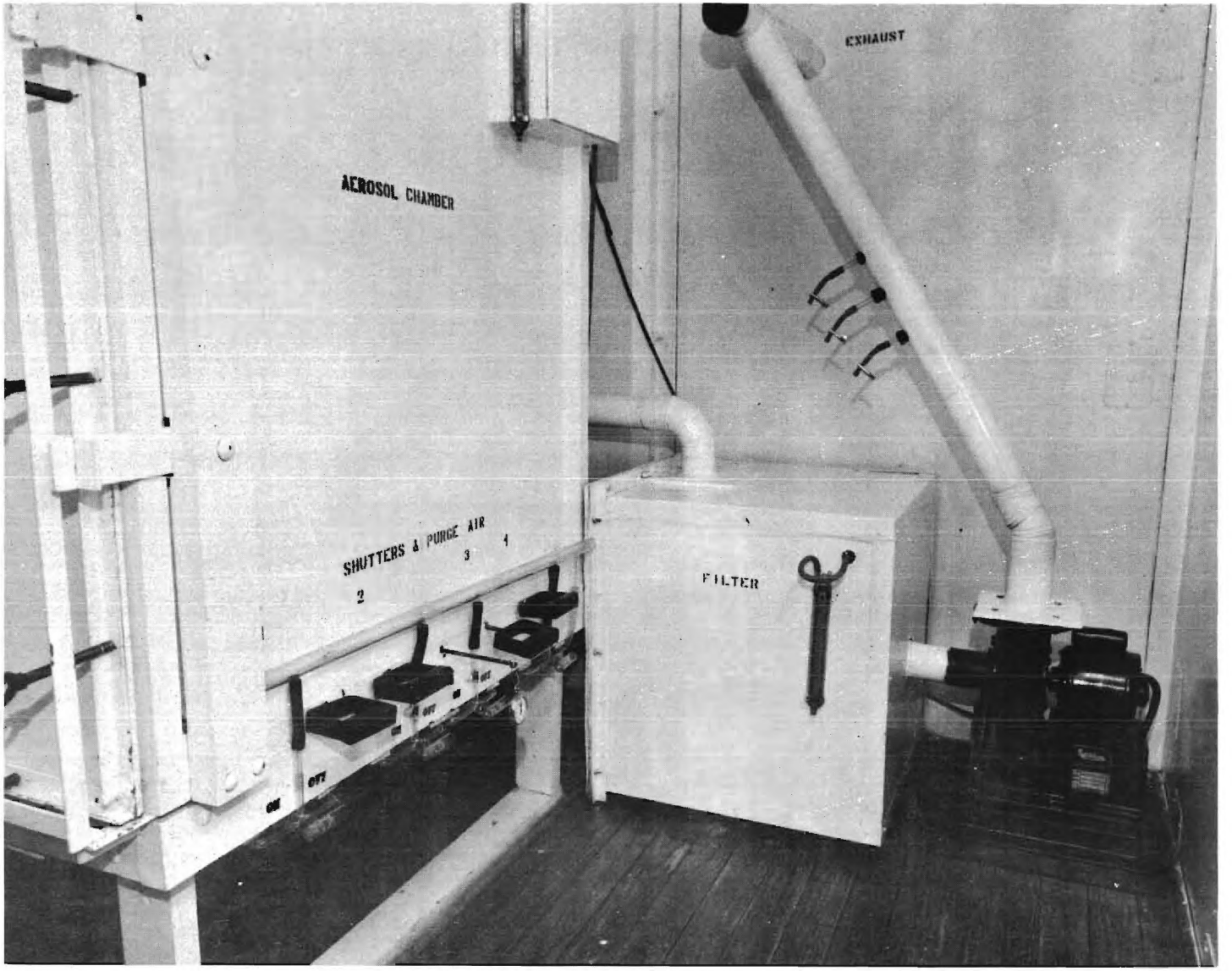


Figure 9. Side View of Aerosol Chamber.

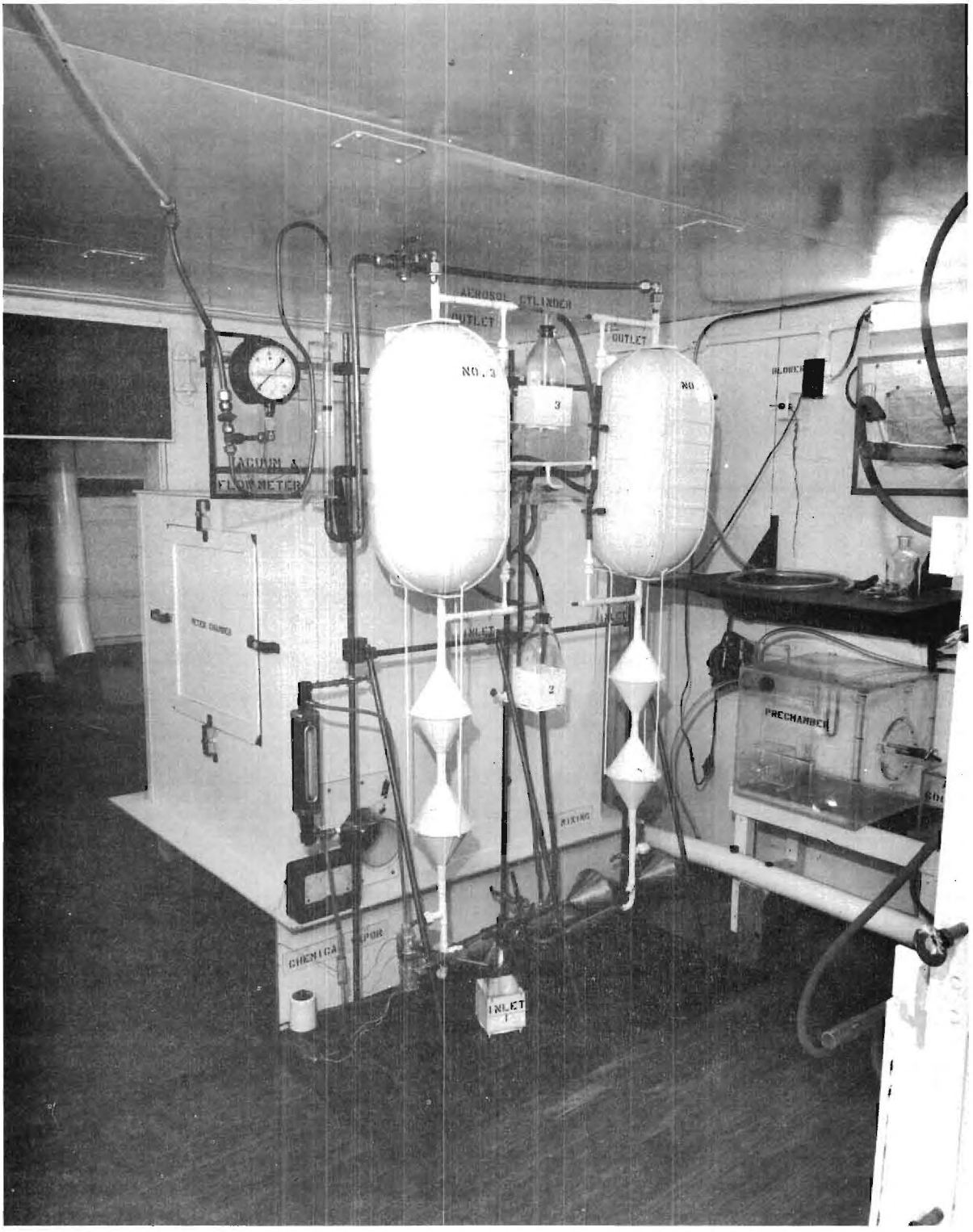


Figure 10. Aerosol Cylinders.

other. The source of the aerosol used in the cylinders is the same as that for the chamber, and an orifice restriction in the line leading from the prechamber to the horizontal mixing funnels maintains the same backpressure in the prechamber as is the case when using the chamber. Filtered room air is drawn into the horizontal mixing section along with the aerosol and is then directed by a valve into one or the other of the basic cylinder units. Where very low-humidity conditions are desired, the make-up air may be passed through a container of silica gel. The chemical vapor is introduced into the system just beyond the entrance of the aerosol and the main air and is pulled upward with them and mixed in the vertical mixing section prior to entering the cylinder. The mixture of vapor and aerosol passes through the cylinder, the flowmeter, the critical orifice which controls the flow rate, and then out through the vacuum line. Sampling tubes are provided at the inlet to the vertical mixing section just prior to the point of introduction of the chemical vapor, at the inlet to the cylinder, and at the outlet of the cylinder. In order to maintain pressure and volume balances in the system, a volume of air equal to that removed in sampling is constantly withdrawn from the system during the nonsampling periods.

The components of the chemical-vapor-generating unit for the aerosol cylinders are shown in Figure 11. In this figure, at the left is the small diaphragm pump (60 to 1200 cc/min) which forces air from the silica gel tube through the midget impinger containing the compound to be vaporized. Also on the left, an impinger is shown standing beside the heating unit containing another impinger. The temperature of the heating unit is adjusted by varying the voltage delivered to the heating unit until the desired temperature is indicated on the microammeter (shown in the center of Figure 11). This microammeter is connected to a copper-constantan thermocouple imbedded in the heating unit. The chemical-vapor generator used for setting up concentrations of compounds in the meter

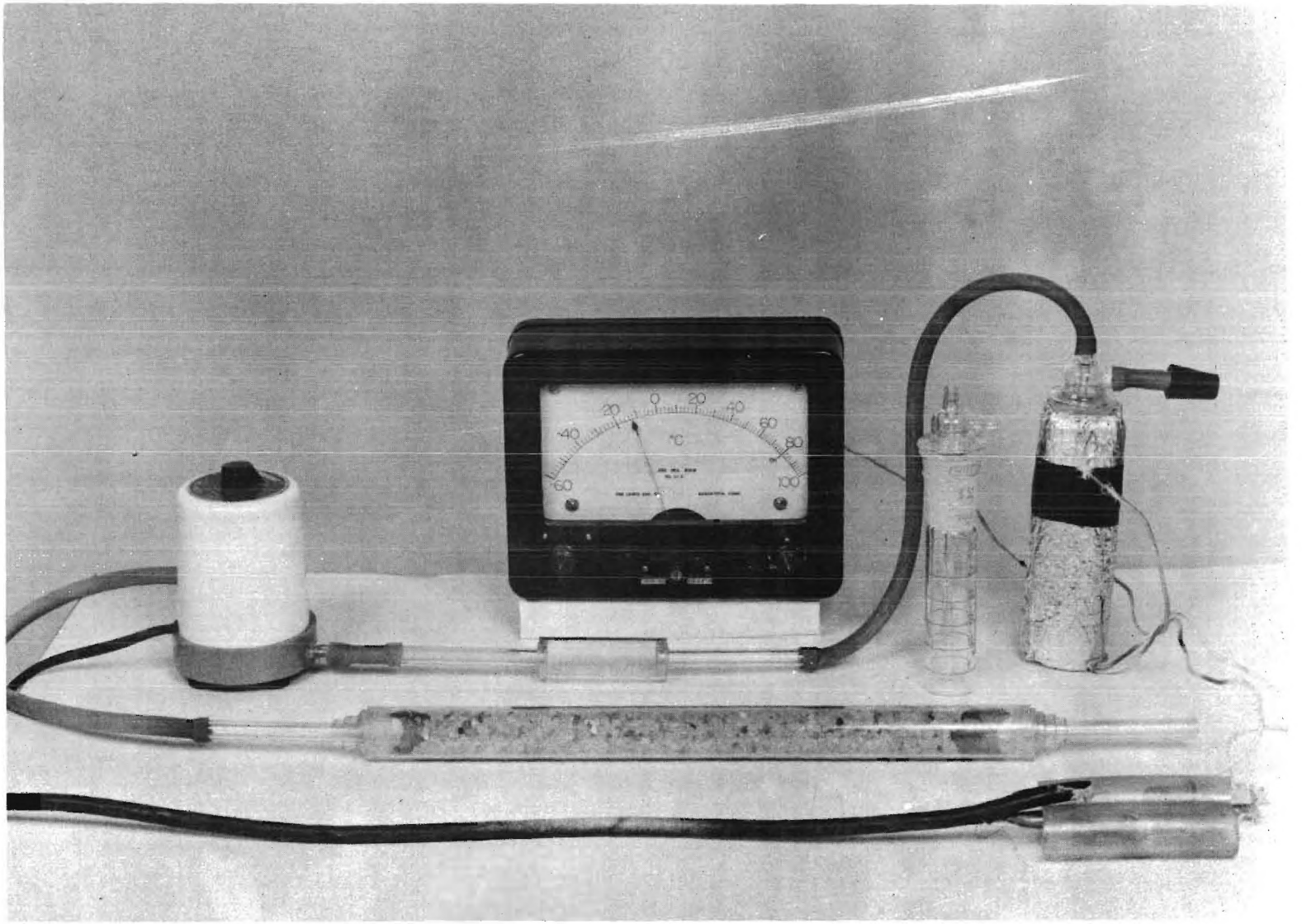


Figure 11. Small Chemical Vaporizer.

chamber prior to use in the aerosol chamber is simply a larger model of the unit shown in Figure 11.

The method of cleaning the aerosol cylinders is illustrated in Figure 12. Live steam is run through the cylinder, followed by dry air. The cylinder is then returned to the workroom for thermal equilibration prior to reuse.

Although the majority of the experimental work carried out on this project was done in the insulated workroom with the equipment described, the culture room and general laboratory facilities were essential adjuncts. A general view of the exterior of the workroom, balcony, and culture room is shown in Figure 13. A view of the transfer table within the culture room is given in Figure 14. A general view of the laboratory (separated from, but convenient to, the other facilities) is shown in Figure 15.

B. The Production of the Standard Bacterial Aerosol

A standard, reproducible bacterial aerosol is a prime necessity in studies of experimental aerobiology. The output of the prechamber system (described previously in this chapter) has constituted the standard bacterial aerosol in all the studies reported herein. The various precautions taken in the production of this aerosol were necessary in order to minimize two different types of variation; minute-by-minute changes occurring within each day's runs and variations possibly occurring from day-to-day. The combination of mechanical controls and cultural controls (described in detail in Chapter IV of this report) has maintained both of these variations at a minimum. A description of the individual particles making up the standard bacterial aerosol is found in Chapter V under "The Size and Nature of the Air-borne Particle."

1. Variations Within Runs

The operations involving the use of the aerosol cylinders for the screening of candidate aerial disinfectants necessitated the continuous operation

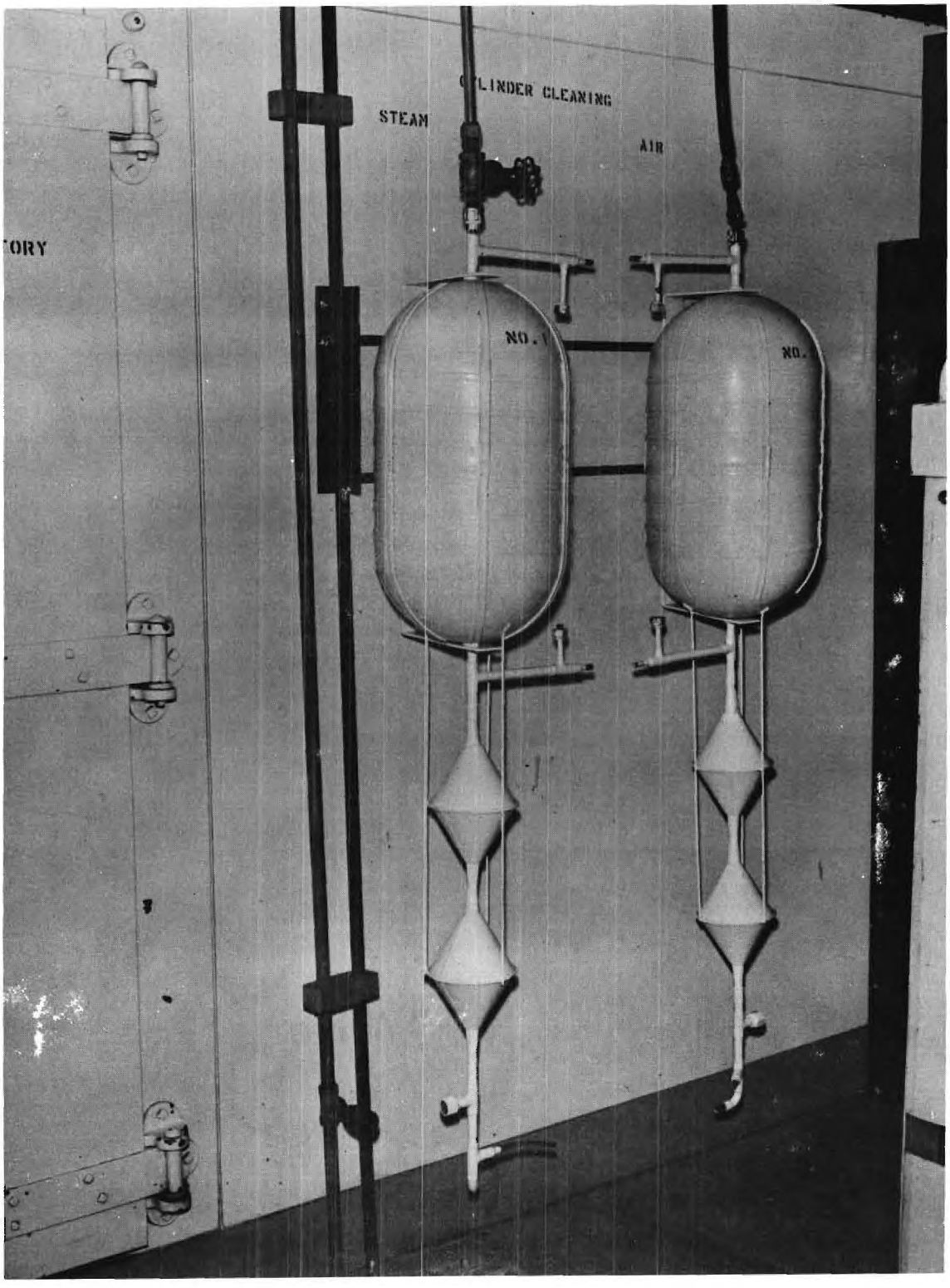


Figure 12. Cleaning Aerosol Cylinders.

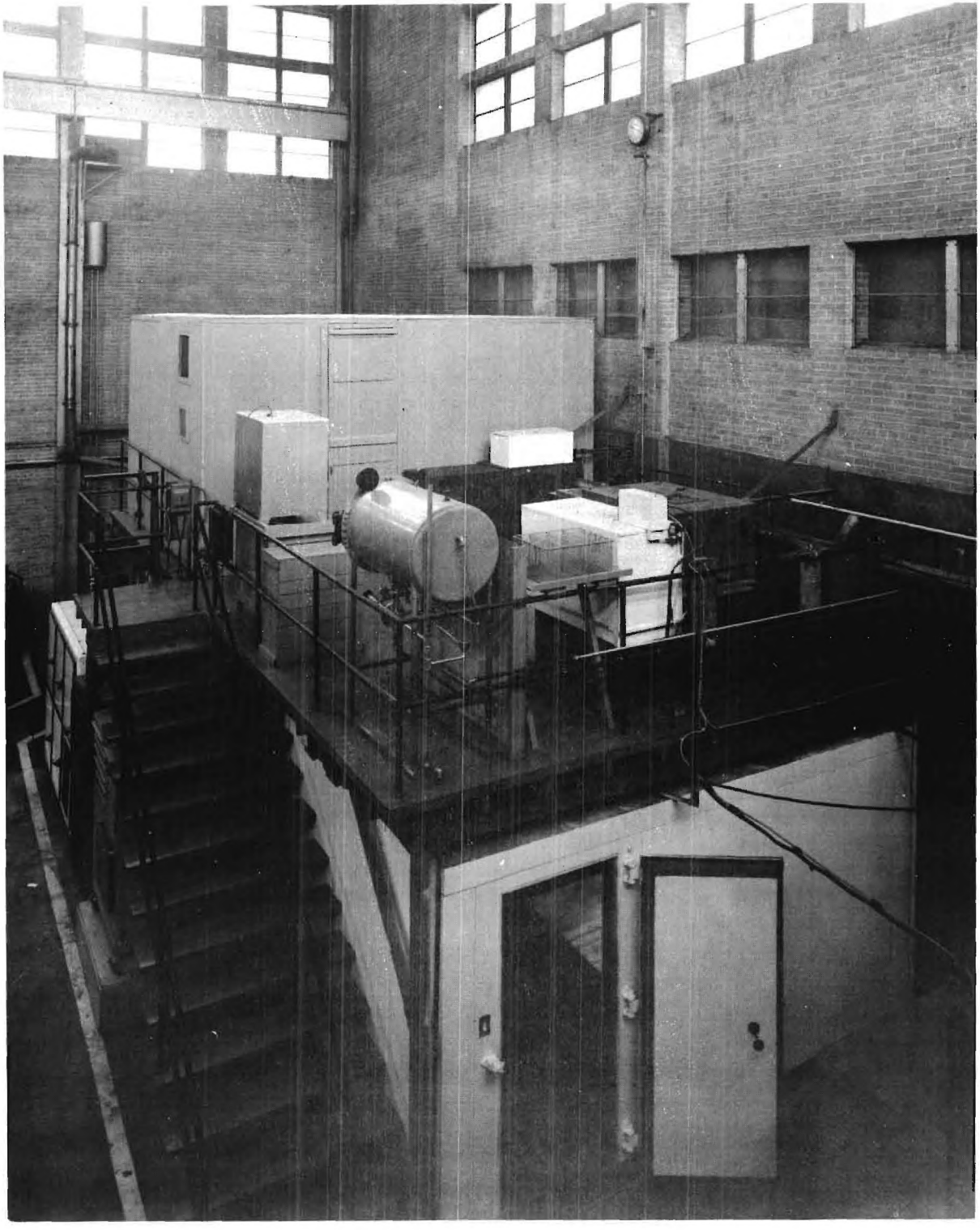


Figure 13. General View of Exterior of Workroom, Balcony, and Culture Room.

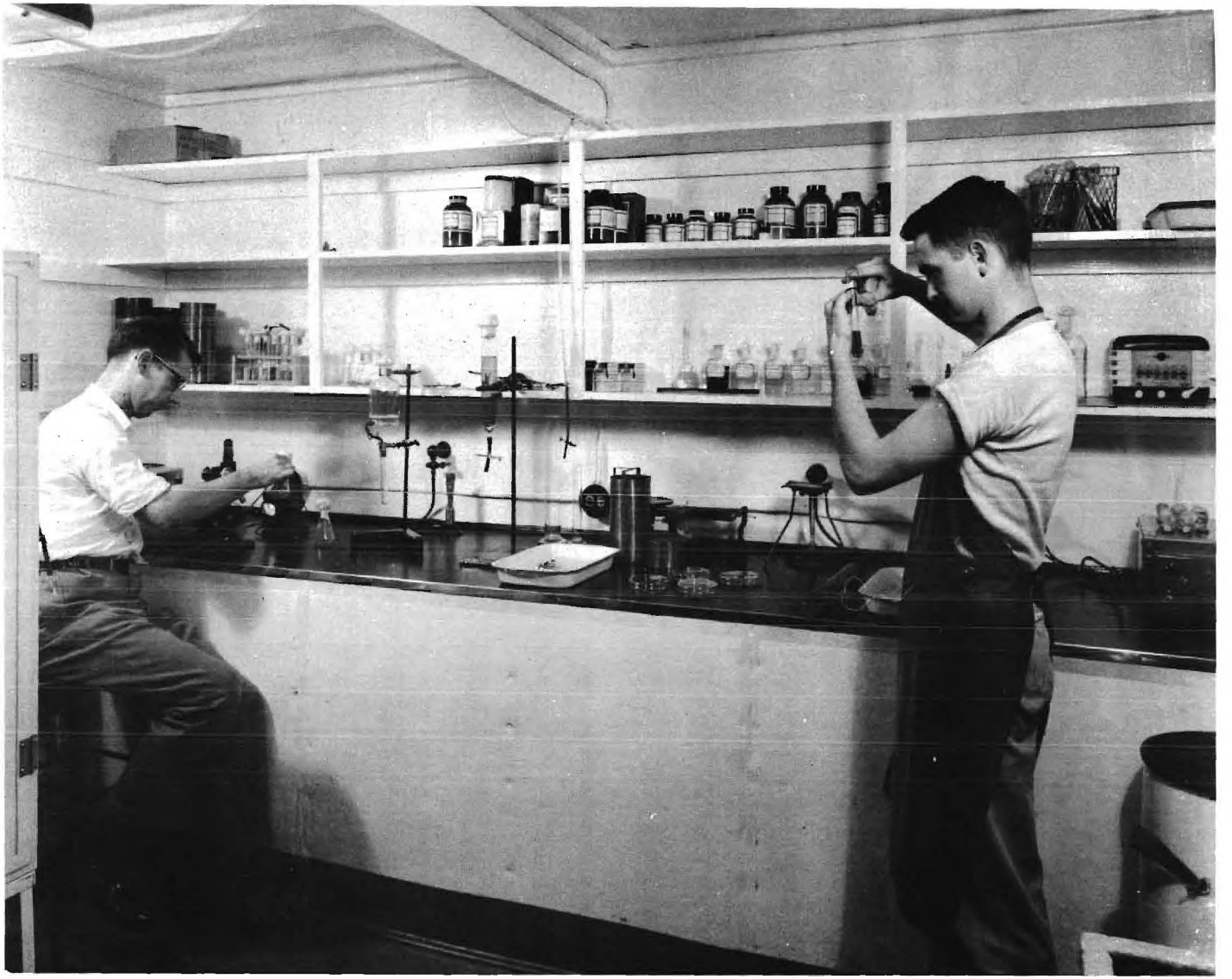


Figure 14. Transfer Table within the Culture Room.

Figure 15. View of the Laboratory.

of the prechamber system for a period of not less than 6 hours each day. Although past experience had shown that the output of the prechamber remained essentially constant during such an operation, a very exact control of the aerial concentration of the bacterial aerosol was desired for the present work, and further investigations were made. It had been established that regulation of the atomizer air supply, control of the temperature of the atomizer bath, and control of the temperature of the room were all required for reasonably constant output by the prechamber. Under these operating conditions, a series of samples were taken at 15-minute intervals during the day. The resultant data indicated a constant output until the middle of the afternoon, followed by a definite increase in concentration of air-borne organisms.

This midafternoon increase in concentration occurred regularly and was finally traced to the operation of a sandblasting machine which drew large volumes of compressed air from the common compressor system. A pressure recorder was installed in the air line, and it was found that the operation of the sandblaster, usually just after lunch, caused an initial drop in the pressure, followed by an average increase, due to the continuous operation of the compressor. The system of reducer valves described in the preceding portion of this section was installed to reduce the variations in pressure to a minimum.

After the installation of the multiple reducers in the atomizer air line, the runs were repeated. It was found that the concentration now showed a definite tendency to rise regularly and gradually during a period of several hours. This difficulty was finally traced to a gradual buildup of numbers of organisms in the atomizer. Apparently, the cyclic fluctuation of pressure in the air lines had, in the past, caused sufficient variations in the flow through the atomizer

so that a continuous mixing of the contents of the atomizer and reservoir was effected. Eliminating the pressure fluctuations had permitted the buildup of an increased concentration of organisms in the atomizer.

In order to eliminate the increased concentration of organisms in the atomizer, the siphon pump was installed to effect continual mixing of the contents of the atomizer with those of the reservoir. The test runs were then repeated, and a vastly improved situation was found.

Typical runs from the above-mentioned series of tests are shown in Table I. In this table, "Condition 1" refers to the original operating conditions; "Condition 2" refers to the operating conditions after the installation of the multiple regulators in that atomizer air line; and "Condition 3" refers to the final arrangement with multiple regulators and the siphon pump. Operating under these various conditions, the last named condition yielded the most consistent concentration of air-borne organisms during the several hours of operation. All the data in Table I are derived from the results of samples of 1-minute duration; such variations as are found in this table under "Condition 3" are probably due to the shortness of the sampling period and the resultant probability of small errors in timing.

In the course of these studies, counts of total and viable organisms were made from the atomizer and from the reservoir prior to the runs and at the end of the runs. The beneficial effect of the siphon pump in effecting mixing between the atomizer and the reservoir is readily apparent when such data, as shown in Table II, are examined. Without the pump the concentration of cells, both total and viable, in the atomizer increased markedly. This concentration effect was completely overcome by the mixing action of the pump.

TABLE I

VARIATION IN NUMBERS OF VIABLE AIR-BORNE ORGANISMS ISSUING FROM PRECHAMBER DURING OPERATION AT 68° F, 60 PER CENT RELATIVE HUMIDITY

Time	Average Number of Viable Organisms Per Liter of Air x 10^{-4}		
	Condition 1	Condition 2	Condition 3
10:30 AM	---	252	---
45	---	208	---
11:00	---	249	264
15	286	300	260
30	288	239	340
45	253	294	314
12:00 Noon	258	349	294
15	250	410	284
30	250	409	225
45	257	398	330
1:00	168	492	300
15	266	445	265
30	216	535	284
3:00	455	510	---
15	450	605	---
30	470	593	270
45	483	690	320
4:00	565	415	286
15	545	555	280

2. Variation from Day-to-Day

In examining the output of the prechamber for variations which might occur from day-to-day, the most readily available data were those obtained from samples taken at the inlet to the aerosol cylinders. A large number of such samples were taken over a period of several months, all operations being identical except that the relative humidity was held at various conditions during these runs. The results of the samples taken following equilibration with the desired relative humidity conditions were accumulated for these various conditions. All numbers of viable air-borne organisms were calculated to an original culture count of 100×10^7/ml and were expressed in terms of concentration of viable organisms per liter of air issuing from the prechamber. The resultant averages, their respective standard errors, the number of individual observations, and the coefficients of variation are shown in Table III.

Examination of the information included in Table III indicates that there are some significant differences among the average number of viable organisms for several of the conditions of relative humidity. Subsequent analysis of this factor showed that this is probably due to a varying ability of the organisms to survive the change from near saturation in the prechamber to the particular relative humidity condition of the run. This varying ability to survive equilibration to rapid change in relative humidity is probably caused by the response to varying rates of drying. This is discussed in detail in Chapter V of this report.

The data in Table III show that, for any particular relative humidity, the coefficients of variation are reasonably small, including as they do all of the variations produced in operation, sampling, diluting, plating, and counting.

TABLE II

EFFECT OF SIPHON PUMP ON NUMBERS OF ORGANISMS IN ATOMIZER FOLLOWING SIX HOURS OF OPERATION

Operating Conditions	Number of Organisms per Milliliter of Fluid x 10^{-7}					
	Initial Culture		Atomizer At End of Run		Culture Reservoir At End of Run	
	Total†	Viable	Total†	Viable	Total†	Viable
Pump Off	190	97	348	152	204	100
Pump On	200	86	200	83	212	82

† Total counts made by enumerating all cells in a Petroff-Hauser counting chamber

TABLE III

NUMBERS OF VIABLE AIR-BORNE ORGANISMS ISSUING FROM PRECHAMBER AFTER EQUILIBRATION WITH ROOM AIR OF VARIOUS HUMIDITIES AT 68° F

Per Cent Relative Humidity	Average Number† of Viable Organisms per Liter of Air x 10^{-4}	Standard Error of the Average	Number of Observations	Coefficient of Variation
20-25	378	11.5	43	20.0
26-34	296	11.2	11	12.5
45-55	325	9.4	97	28.0
58-62	282	7.1	9	7.5
64-66	232	14.0	12	21.0
73-77	252	12.3	17	20.0
78-82	274	8.9	69	27.0
88-92	245	7.4	6	7.5

† Corrected to an original culture count of 100 x 10^{7}/ml.

That is to say, these data show, that at any time, it is possible to produce essentially the same bacterial aerosol in approximately the same concentrations, over a period of several months. Such a situation increases confidence in results obtained during such an interval, as well as simplifies the routine bacteriological procedures involved in diluting samples for counting.

C. Characteristics of the Main Aerosol Chamber

The main aerosol chamber, previously described, was designed in order to provide a device for studying, in detail, the behavior of bacterial aerosols. The requirements for such a device were given in an earlier report (Progress Report E-141-C) and are as follows: the chamber should be large enough to permit sampling without seriously disturbing its contents, yet small enough to be housed in most laboratories; the chamber and its environment should have adequate temperature and humidity controls; the chamber should operate equally well either statically or dynamically; provision should be made for obtaining both settling and air samples; in operation, its characteristics should be reproducible; the bacterial aerosol should be evenly distributed throughout the chamber; and, in operation, it should be possible to account for all the bacteria dispersed into the chamber. The present chamber embodies all of these characteristics.

1. Aerosol Distribution Within the Main Chamber

A study was undertaken to determine the distribution of the bacterial aerosol particles within the 4-foot-cube main aerosol chamber operated under dynamic condition of 60 cfm of air. The design estimate of the chamber equipped with a diffusing head for the incoming air-aerosol mixture indicated that a high degree of dispersion and homogeneous distribution throughout all points within the chamber could be expected. To determine the distribution of the bacterial

aerosol within the chamber, a vertical series of five apertures was made in the center of one wall of the chamber, and a similar vertical series of five apertures was made in one quadrant of the same wall. Reference to Figure 5 will show the arrangement of the apertures and their numbered designations. Straight glass-tube probes, 0.25-inch internal diameter, were inserted into these apertures, through rubber stoppers, to hold them firmly in place. The probe intruded into the chamber approximately 4 inches and was separated from the vertically adjacent probes by an interval of approximately 7.5 inches. This was the standard physical arrangement of the sampling probes. In the sampling of any of the aerosol clouds to determine the distribution of the cloud, various lengths and positioning of probes were used and have been so designated in reporting the results. Unless otherwise specified, all probes are in the standard position.

References to Tables IV and V show the results of concomitant samples taken from various points within the chamber during dynamic operation. The positions of the probe are such as to allow for adequate sampling of the interior of the chamber, as well as the surface of the chamber wall and the region of wall-floor junction. The total volume of aerosol sampled is approximately the same in each experiment, with the notable exception of the 5-liter-per-minute samplers exposed for 35 minutes. These data reflect a very good distribution of the bacterial aerosol throughout all portions of the chamber, and the variations encountered are within acceptable limits of error.

Preliminary studies of aerosol clouds composed of various smokes or bacteria demonstrated the characteristic of a cloud to maintain an integrated form resisting dispersion and dilution when introduced into a chamber. Visualization of the cloud by a Tyndal beam showed that the pattern of movement is imparted

TABLE IV

AEROSOL DISTRIBUTION WITHIN THE MAIN AEROSOL CHAMBER†

Probe No.	Sampler Capacity (l./min)	Concentration of Bacteria Per Liter of Air†† Experiment No.1 Run 1	Experiment No.1 Run 2	Experiment No. 2 Run 1	Experiment No. 2 Run 2
1†††	5	2780	3100	2760	2080
2	1	3000	3500	2610	2210
4	1	3130	2840	2710	2440
5††††	5	2780	4000	2120	1875
8	1	3280	4550	3100	----
8	2	----	----	----	3420
	Average =	2994	3598	2660	2405
	σ =	219	689	354	601
	C.V. =	7.3	19.2	13.3	25

† Culture: S. marcescens, ATCC 274, E-R
Atmosphere: dry bulb 76° F, relative humidity 70 per cent
Sampling Period: 35 minutes
Sampling Medium: 0.2 per cent gelatin solution, buffered

†† Concentration of bacteria per liter of air not corrected to 100 x 10^7/ml original culture.

††† Orifice of sampling tube contiguous to chamber wall.

†††† Orifice of sampling tube contiguous to chamber floor.

by the nature of the introducing orifice, and, in a condition of nonturbulent flow, the entering cloud may be followed for a considerable period of time within the chamber. Devices of various designs for disrupting the cloud by producing turbulent mixing were interposed in the entering conduit, and/or within the

TABLE V

AEROSOL DISTRIBUTION WITHIN THE MAIN AEROSOL CHAMBER†

Location of Sampling Probe	Probe No.	Run 1 (conc/l.)	Run 2 (conc/l.)	Probe No.	Run 1 (conc/l.)
2 feet from wall	1	2890	2640	6	1750
1 foot from wall	2	2420	2860	7	2720
2 inches from wall	3	2960	2810	8	2700
2 inches from wall and floor	4	2700	3000	9	1960
2 feet from wall	5	2570	2900	10	2580
Average =		2708	2842		2342
σ =		223	133		454
C.V. =		8.2	4.7		19.4

† Capacity of Impinger Sampler: 5 liters per minute.

Sampling Period: 5 minutes.

Sampling Medium: 1.6 per cent brain-heart infusion - 0.2 per cent gelatin solution, buffered

Culture: S. marcescens, ATCC 274, E-R.

Atmosphere: Dry bulb 40° F, relative humidity 57 per cent.

chamber. The failure to disperse the cloud amply demonstrated the resistance of the cloud to any dilution effects. Stirring apparatus within the chamber would be undesirable because it would not provide the random distribution under dynamic conditions which are necessary but would produce flow patterns of palpable velocity. The qualities of the observed cloud present a fundamental aspect of chamber design requirements to be met by any chamber system providing satisfactory conditions of random samples during dynamic or static operating conditions.

The present design of the main aerosol chamber incorporating the Anemostat diffusion head has resulted in a chamber which provides a continuously operating system, producing a homogeneous dispersion of a bacterial aerosol cloud. During months of operation, the system has shown a high degree of reliability, and it has provided the basic component in the study of microorganisms, sampling devices, and related techniques of experimentation.

2. Dynamic Filling and Emptying Characteristics of the Main Chamber

The inimical effect of different atmospheres on air-borne bacteria is expressed as k in this report and is obtained from the relationship of the log of the initial number of organisms (N_0) minus the log of the final concentration of organisms (N_1) divided by the time (t) of exposure. In the operation of the main aerosol chamber, a 4-foot cube, the factor t is equal to the time required for the passage of 99 per cent of a bacterial aerosol cloud through the chamber, and it is determined by the rate of flow of the aerosol. In the dynamic operation of the main aerosol chamber, the rate of flow has been 60 cfm. To determine the value t for the chamber operated at 60 cfm, an aerosol of S. marcescens ATCC 274, E-R, was introduced into the chamber for not less than 15 minutes to insure complete equilibration before brain-heart-gelatin, critical-orifice, impinger samplers were employed to determine the bacterial concentration. At time t, the bacterial aerosol was by-passed from the chamber, and impinger samplers of 1-minute exposure were started. As the chamber was emptied, samples were taken in 1-minute sequences, $t_1 \ldots t_5$. All samples were taken from the chamber exit.

The results are shown in Table VI and indicate that the time required for the passage of 99 per cent of the aerosol cloud through the chamber, under the dynamic conditions stated, is 5 minutes. To compare the experimental data with

TABLE VI

DYNAMIC EMPTYING CHARACTERISTICS OF THE MAIN AEROSOL CHAMBER

Per cent† Residual Aerosol	Time (min)	Bacterial Concentration Per Liter of Air			
		Run 1		Run 2	
		Actual	Theoretical	Actual	Theoretical
100	0	2695	2695	6470	6470
39	1	3021	1050	5250	2520
15	2	1265	403	1650	973
6	3	485	161	860	389
2.4	4	167	64	260	155
1.5	4.5	----	40	----	97
0.9	5	80	13	90	58

† Per cent residual aerosol = $100\ (e^{-\frac{f}{v}t})$
Where f = chamber air-flow rate = 60 cfm and
v = chamber volume = 64 ft^3.

the expected theoretical disappearance of the aerosol, the formula expressing the per cent residual aerosol at any time t is used. This formula is

$$\text{per cent residual aerosol} = 100\ (e^{-\frac{f}{v}t})$$

where f = chamber flow rate, cfm,

v = chamber volume, ft^3, and

t = time in minutes.

This formula is based on the ventilation characteristics of a gas and presumes perfect and instantaneous mixing or diffusion. It will be noted that the experimental and theoretical results are in acceptable agreement relative to numerical

magnitude but are approximately 1 minute out of time phase, e.g., the corresponding experimental value is 1 minute later than the theoretical value. This result is derived from the fact that a bacterial aerosol cloud does not possess the comparable high rate of mixing and diffusion characteristics of a gas and from the fact that the impinger sample of $\underline{t}_1$, started at the same time as the aerosol was by-passed, was actually sampling an undiluted aerosol cloud. This 1-minute lag of the experimentally derived emptying characteristic of the chamber does not invalidate the determined value of 5 minutes for the time required for the passage of 99 per cent of an aerosol cloud through the chamber operated at 60 cfm.

The filling characteristics of the main aerosol chamber were similarly determined but in reverse order. Into the dynamically operating chamber, a bacterial aerosol was introduced and critical-orifice impinger samples were taken to monitor the increasing concentration of aerosol within the chamber. From the ventilation equation, it is apparent that the chamber concentration, at first, rises rapidly and then approaches a constant value at infinite time. The experimental data, Table VII, confirm this rapid change. The initial high rate of change (61 per cent) during the first minute of introduction into the chamber presents a considerable problem in the collection of a sufficient number of organisms during very brief intervals of time using the 1-liter-per-minute sampler. During the initial phase, samplers were operated for a single period of 3 minutes and, subsequently, for periods of 1 minute each. Although the initial phase of the filling characteristic was obscured to a degree, the first and subsequent samplers indicated that the chamber exhibited an acceptable performance in accordance with the expected filling characteristics.

TABLE VII

DYNAMIC FILLING CHARACTERISTICS OF THE MAIN AEROSOL CHAMBER

Theoretical Performance			Experimental	
Per Cent† Aerosol	Time (min)	Air (conc/l.)	Time (min)	Air (conc/l.)
0	0	0	0	0
61	1	3840	0-3	3240
85	2	5350	3-4	4650
94	3	5930	4-5	5700
97.6	4	6150	5-6	6450
98.5	5	6200	6-7	6450
99.1	6	6300††		

† Per cent aerosol = $100\ (1 - e^{-\frac{f}{v}t})$
where $\frac{f}{v}$ = chamber flow rate = 60 cfm,
$\frac{v}{t}$ = chamber volume = 64 ft^3, and
$\frac{t}{}$ = time in minutes.

†† Average concentration of bacteria per liter of air after approximately 10 minutes of chamber equilibration.

In order to determine whether or not the initial lag exhibited during the emptying of the chamber is characteristic of the aerosol cloud or of the chamber itself, data were obtained on the filling characteristic of the chamber using water vapor. From the mathematical expression of theoretical emptying and filling characteristics, it is evident that determined values are convertible. Water vapor, as a gas, should diffuse instantly, and any significant variation from the theoretical results would indicate that the chamber is not operating properly. In this experiment, water vapor was introduced into the chamber by

atomization at such a rate as to give a final concentration equivalent to about 80 per cent relative humidity. The room air was maintained at about 20 per cent relative humidity. Under these conditions, the output of the thermal conductivity cell could be set at zero for the ventilation of room air through the chamber, and, after prolonged atomization of the water into the chamber, an output signal of about 5.0 mv (one-half full scale) could be obtained. These conditions of operation were chosen to obtain maximum sensitivity of the thermal conductivity cell without ever approaching a limiting value of saturation. The thermal conductivity cell output is directly proportional to the amount of water present in the air.

Several runs were performed under the conditions outlined above, and agreement was obtained among the resulting curves plotted on the Esterline-Angus recorder. These data are shown in Table VIII, as are the theoretical values for per cent equilibrium. In general, there is reasonable agreement between the actual and the theoretical values. It is concluded that the 99 per cent equilibration time for the chamber is as calculated and is in agreement with the data obtained from the study of the emptying characteristics of the chamber employing bacterial aerosols.

In summary, the performance of the dynamically operated aerosol chamber has demonstrated the soundness of design in providing an apparatus for the production and containment of a homogeneous bacterial aerosol cloud. The reliability of production and dissipation as well as the dispersion of the aerosol cloud within the chamber provides a primary basis of confidence in the subsequent studies on air-borne bacteria.

TABLE VIII

DYNAMIC FILLING CHARACTERISTICS OF THE MAIN AEROSOL CHAMBER USING WATER VAPOR AS INDICATOR

Time (min)	Thermal Conductivity Output† (mv)	Per Cent Equilibration	
		Actual	Theoretical††
0.0	0.0	0.0	0.0
0.625	2.7	49.0	44.6
1.25	4.0	73.0	69.0
1.875	4.5	82.0	84.7
2.5	5.0	91.0	90.4
3.75	5.3	96.5	97.1
5.0	5.5	100.0	99.1
6.25	5.5	100.0	99.7

† The thermal conductivity cell output was adjusted to zero prior to the introduction of water vapor into the chamber.

†† Per cent equilibration = $100 \left(1 - e^{-\frac{f}{V} t}\right)$
where f = chamber flow rate = 60 cfm,
V = chamber volume = 64 ft^3, and
t = time in minutes.

D. Characteristics of the Aerosol Cylinders

Although the main aerosol chamber has proven to be an excellent chamber component in the system for the production and containment of bacterial aerosol clouds under continuously operating conditions, there are inherent disadvantages in its use as an apparatus for the screening of chemical compounds for aerial disinfection. This disadvantage is primarily one of chamber size and operational

detail. Following the establishment of a standard bacterial aerosol cloud and its subsequent treatment with a chemical compound, it is necessary to isolate the chamber component for meticulous cleaning of all surfaces in order to remove traces of the chemical tested. Although this is quite feasible with the chamber proper, such a procedure is time consuming, and, furthermore, the permanent installation of conduits presents a problem. However, where the detailed study of previously selected and tested chemical compounds is desired, this procedure is not excessive for the aims and value of such detailed study.

The above requirements indicated the need for a much smaller chamber, one easily manipulated and having the same general characteristics of the aerosol chamber. In general, these characteristics are: uniformity of distribution of the bacterial aerosol throughout the system; a measurable detention time; capability of operating at various temperatures and relative humidities; and size--large enough that the sampling operation does not produce dilution errors. The low-pressure, nonshatterable, oxygen cylinder used by aviators during high-altitude flying was found to meet the requirements of such a chamber almost exactly. These cylinders are made of stainless steel, are 12 inches in diameter, having a straight cylindrical midsection 12 inches in length, and are 24 inches in over-all length, the ends being dome-shaped. The volume of the cylinder is about 35 liters. The cylinders may be used separately or paired in series, depending upon the detention time desired. The single- or dual-cylinder system is operated at 30-liters-per-minute air flow, a flow which approaches the capacity of the available vacuum pumps when operating across an orifice at the critical-pressure ratio. This method of exactly controlling the flow is a most satisfactory one and is to be preferred whenever practical. The system is operated

with an upward air flow, and the 30-liters-per-minute flow provides sufficient upward velocity to maintain the aerosol particles air-borne at all times.

The determination of the aerosol detention time for the cylinders was necessary because the k factor for the cylinders is determined in the same manner as for the aerosol chamber, e.g., log N_0 - log N_1 divided by the detention time. The emptying characteristics for the dual-cylinder system--a system of two cylinders connected in series--were determined and are reported in Table IX. The general characteristics of the dual-cylinder system appear to agree with the theoretical considerations of a chamber of these dimensions. In the time sequences later than 5 minutes, the values obtained differ from the theoretical values, the actual emptying rate of the cylinders showing an increase. This increased rate of emptying may be caused by a parabolic profile of the withdrawing aerosol cloud which is exhibiting terminally a more rapid dilution and removal. Also, it is to be recalled that the cylinders are arranged in series and, although considered as a single chamber, the system may possess some characteristics peculiar to this arrangement. Several measurements have indicated an emptying time of slightly over 7 minutes, and, for ease of computation, 7 minutes was accepted as the aerosol detention time for the dual-cylinder system.

The routine, chemical-screening tests were conducted employing only one cylinder at a time. The detention time was estimated to be approximately one-half of the dual-cylinder system's time and, to verify this, the dynamic filling characteristics of the single-cylinder system was determined directly using a bacterial aerosol cloud and, also, water vapor. Since the essential factors of the ventilation formula are common to both the filling and emptying phenomena, the data for filling or emptying are directly comparable.

TABLE IX

DYNAMIC EMPTYING CHARACTERISTICS OF THE DUAL CYLINDER

Per Cent† Residual Aerosol	Time (min)	Bacterial Concentration Per Liter of Air			
		Run 1		Run 2	
		Actual	Theoretical	Actual	Theoretical
100	0	-----	50300††	-----	57300††
65	1	33200	33200	37800	37200
42	2	25700	21100	33700	24000
27	3	14300	13600	16000	15450
18	4	8700	9050	9800	10300
12	5	5000	6030	4750	6850
7	6	2340	3520	2930	4000
5	7	1500	2510	1420	2860
3	8	670	1510	700	1715

† Per cent residual aerosol = $100\ (e^{-\frac{f}{v}t})$
where f = chamber air-flow rate = 30 liters per minute, and
v = chamber volume = 68.8 liters.

†† Values derived from experimental values at 1 minute.

In determining the dynamic filling characteristics of the single-cylinder system, water vapor was introduced into the inlet, and the rise in concentration of water vapor at the outlet was recorded directly from the response of a thermal conductivity cell located at the outlet. The data resulting from the use of water vapor as an indicator, as reported in Table X, appear to be in agreement with the theoretical values. The data also shows a tendency of increased rate of equilibrium as a function of time, as was seen in the emptying characteristics of

TABLE XI

DYNAMIC FILLING CHARACTERISTICS OF THE SINGLE CYLINDER USING WATER VAPOR AS INDICATOR

Time (min)	Thermal Conductivity Output† (mv)	Per Cent Equilibration†† Actual	Per Cent Equilibration†† Theoretical
0.0	0.0	0.0	0.0
0.25	0.80	21.0	20.0
0.5	1.55	40.0	35.0
1.0	2.65	68.5	58.0
1.5	3.3	85.5	73.0
2.5	3.75	95.5	89.0
4.0	3.8	100.0	97.0

† The thermal conductivity cell output was adjusted to zero prior to the introduction of water vapor into the cylinder.

†† Per cent equilibration = $100\ (1 - e^{-\frac{f}{v}t})$
where $\frac{f}{v}$ = chamber flow = 30 liters per minute, and
$\frac{f}{v}$ = chamber volume = 34.4 liters.

the dual-cylinder system. The possible explanation given previously for this behavior is applicable to the filling characteristics. The results obtained when using a bacterial aerosol cloud as an indicator, as reported in Table XI, are not as helpful because of the exponential increment of the admitted cloud-air mixture and the minimum practical time of the sampling method. The general results show a basis for accepting the detention time value of 3.5 minutes for the single-cylinder and 7 minutes for the dual-cylinder system.

The results obtained from prolonged usage of this apparatus indicate a high degree of reproducibility of operation. The objectives of the original design

TABLE XI

DYNAMIC FILLING CHARACTERISTICS OF THE SINGLE CYLINDER

Per Cent Aerosol†	Theoretical Performance		Experimental	
	Time (min)	Air†† (conc/l.)	Time (min)	Air†† (conc/l.)
0	0	0	0	0
58	1	121	0-1	42
83	2	173	2-3	123
93	3	194	4-5	195
95	4	198	6-7	206
97	5	204	---	---
99	-	209†††	---	---

† Per cent aerosol = $100 \left(1 - e^{-\frac{f}{V} t}\right)$
where f = chamber flow rate = 30 liters per minute, and
V = chamber volume = 34.4 liters.

†† Concentration of bacteria per liter of air = N x 10^3.

††† Concentration after approximately 15 minutes' equilibration.

have been attained in an apparatus which permits the screening of chemical compounds at a rate greatly in excess of that possible with the main aerosol chamber. Because the cylinder system is much smaller than the aerosol chamber, it has the objectionable characteristics of many small systems, such as sampling dilution effects during operation. The value of the cylinder system may be enhanced by making possible the study of the effects of the atmosphere on several microorganisms in rapid succession, much in the same manner as is used for rapid chemical screening. In this latter regard, advantage can be made of the variable detention time of 3.5 and 7 minutes provided by the single- and dual-cylinder system,

as different organisms may exhibit wide differences in response to the lethal effects of the atmosphere. Longer detention times could be achieved by using additional cylinders in series. These features, together with the facility of cleaning, make this system extemely valuable in the study of air-borne bacteria.

IV. BACTERIOLOGICAL METHODS AND CULTURES

In planning the program of work for this project, the primary test organism, S. marcescens, was selected on the basis of natural occurrence, nonpathogenicity, relatively simple nutritive requirements, and other characteristics which facilitated the use of this bacterium without extraordinary procedures and techniques. The dispersing and collecting media were also selected to simplify the preparation and to satisfy the nutritive requirements of the bacterium. The expenditure of extended effort on this phase of the project was not considered to be of prime import at this particular time.

Several developments strongly emphasizing the nature of the organism and composition of the dispersing, collecting, and plating media in relation to the survival of air-borne organisms under various atmospheric conditions, whether natural or artificial, are presented in this chapter, as well as in other chapters.

A more critical examination of the stock culture than originally was planned was essential. The existence of at least four colonial forms of the various strains of S. marcescens was well recognized, and it had generally been assumed that, if a variant showed no appreciable percentage of any other variant, the culture was stable. However, as it will be pointed out in more detail in later discussions, even slight morphological changes may cause variation in resistance when the bacteria are suspended in the air.

Also, the concept of a secondary environmental effect for air-borne bacteria places primary importance upon the composition of the immediate environment of the bacterial particle in determining the fate of the air-borne bacterium. Beef-extract broth was selected as the dispersing medium because it is a common cultural medium and produces an excellent growth. Though other media might possibly

be more suitable, the primary emphasis of the study was not considered to be the selection of optimum media but rather the selection of suitable and useful media. The original concept of the state of the air-borne organisms held by the personnel of this project was that the single organism in an air-borne particle (as in these studies) is covered by only a thin layer of material derived from the solids of the substrate from which it is atomized. However, evidence that the air-borne bacterium is surrounded by a relatively thick layer of nonliving residual substrate material which forms the actual immediate environment of the bacterium indicates that the actual response of the organism may be obscured by effects peculiar to the presence of the accompanying material. This gives great importance to the nature of the substrate from which the organisms are atomized.

The same factors in selecting the dispersing medium also applied to the original selection of the collecting fluid used in the impinger samplers and the plating materials. Evidence obtained in instances where the organisms may have suffered varying degrees of injury shows that enriched media may be required to demonstrate these attenuated organisms.

The bacteriological methods and procedures used in the work covered by this report are presented in detail in the following sections.

A. General Methods

Standard bacteriological procedures were adhered to in the production of the bacterial test organisms. The bacterial specimens were obtained from the American Type Culture Collection. Transfers were then made into 0.3 per cent beef-extract broth, 60-ml volume, and incubated at 30° C. Serial transfers were subsequently made into beef-extract broth at regular intervals of 45 to 48 hours. Four or five transfers were made before using the culture in tests. Thereafter,

the culture was maintained in beef-extract broth and transferred at the time intervals stated. Stock cultures were maintained in nutrient agar butt tubes with a surface overlay of mineral oil by storing in the refrigerator (5° C). Subsequent culturing of the microorganism in beef-extract broth, according to the routine method described, demonstrated the stability of the culture as determined by $\underline{k}_t$ values under standard atmospheric conditions.

Studies of the growth curve of the organism in beef-extract broth at 22° C showed the maximum stationary phase to occur at approximately 40 hours. The age of the test organism used in the production of aerosol clouds was between 40 and 48 hours, and it was found to provide a physiologically and numerically constant culture for daily use.

All media were standard Difco preparations (Difco Laboratories). Glassware used in the bacteriological procedures was cleaned in a hot detergent (Institutional X, Proctor and Gamble) solution, rinsed in tap water, and finally rinsed in distilled water. All water used in making serial dilutions and in preparation of bacteriological media was first distilled and then passed through a mixed-bed, ion-exchange column (IRA 400 and IR 120, Rohm and Haas).

1. Plating Media

Enumeration of viable bacterial cells was made according to routine bacteriological procedure using solid media. During the early states of this work, nutrient agar was accepted as a generally standard medium suitable for the organisms being used. This medium produces countable colonies within 30 to 40 hours. As a result of testing several solid nutrient media, with the objective in mind of reducing the period of incubation of the bacteria before the counting of the colonies, a buffered, sodium chloride, tryptone-glucose-extract

agar medium was developed which reduced the necessary incubation time by one-half. The composition of the medium was tryptone-glucose-extract agar (24 grams), sodium chloride (5 grams), anhydrous dibasic sodium phosphate (2.5 grams), and deionized water (1 liter). Inoculated agar plates were incubated at the temperature of 35° to 37° C. This medium was adopted as the standard plating medium.

2. Plating Technique

Upon sampling the bacterial aerosol cloud, the impinger solution was plated directly and/or serially diluted in sterile deionized water for subsequent plating. Aliquots (direct and/or diluted) of the sample were plated in triplicate for determination of viable cell numbers. The culture medium (tryptone-glucose-extract agar, buffered as previously described) was maintained at 50° C in a water bath before being mixed with aliquots of the sample in Petri dishes of standard size (9 cm x 1 cm). After solidification of the agar, the plates were stored in an inverted position in an incubator at 35° C for approximately 18 hours. At the end of this time, colony growth was advanced sufficiently to permit enumeration with the Quebec colony counter.

B. Test Organism

The test organism used was S. marcescens (Bizio). This bacterium is of a genus naturally occurring in soil, water, and foods and is used frequently in aerosol studies. Bacterium size, nonpathogenicity, relatively simple nutritive requirements and chromogenicity are characteristics which facilitate the use of this bacterium without extraordinary procedures in technique.

A specimen of S. marcescens was obtained from the American Type Culture Collection, Culture No. ATCC 274. This culture was transferred into beef-extract broth and was maintained in broth by serial transfers at 48-hour intervals. For

the purpose of examining and verifying colony-type formation, dilutions of a 48-hour culture were plated into nutrient agar. Incubation was at 30° C for approximately 12 hours and then at 20° C for an additional 12 to 24 hours' growth. This procedure was found to give very good color development of the colonies. Typically circular, thin, smooth, orange-red colonies were selected for propagation in beef-extract broth. This procedure was followed in establishing the test orgainism.

1. Culture Stability

The culture maintained over a period of several months by the procedure described previously provided an orange-red pigmented organism of approximately 0.7 to 1.0 micron in diameter and 1.0 to 2.0 microns in length. The stability of the test bacterium was periodically affirmed under standard conditions of temperature and relative humidity (68° F and 65 per cent, respectively).

The bacterium S. marcescens ATCC 274 is known to be composed of multiple chromogenic and nonchromogenic variants. Under different cultural conditions, these variants exhibit differential stabilities, the pigmented variant showing the greatest stability.

The primary concern with the variants of S. marcescens ATCC 274 in relation to this investigation was the cultural characterization and maintenance of a standard bacterium as correlated with the standard atmospheric conditions mentioned above. This requirement became apparent when, after months of stable performance, the culture began to indicate changes, as evidenced by significant increases in k_t values--a reduced survival capacity under the standard atmospheric conditions. This changed response was at first thought to be a reflection of error in technique or in the mechanics of operation of the aerosol chamber. The problem was

approached with these factors in mind, including the possible physiological change in the test bacterium. Examination and testing eliminated all those factors under consideration except the bacterium, which was then studied in some detail.

2. Characterization of Stable Culture

The original isolation of the test bacterium was made on the basis of chromogenicity, but, with the manifested physiological alteration, a more definitive characterization of the bacterium in relation to the air-borne state became necessary. This primarily concerned the orange-red variant which was the original isolate. However, some work was done with the other color variants of the blood-red, pink, and white variety. Extensive bacteriological studies were beyond the intent of this project, and, since excellent work on this same strain of S. marcescens has been reported in the literature, efforts were restricted to securing information providing a basis for monitoring the test culture.

The current culture showed an aberrant response to the standard atmospheric conditions by a static k_t of 0.059 in contrast to the firm value of 0.034 established over a period of several months. Transfers were made into beef-extract broth from the previously inoculated nutrient agar stock slant which had been refrigerated without mineral oil overlay. The static k_t of the aerosol chamber was determined to be 0.075 showing a similar deterioration of the stock culture. An entirely new specimen of S. marcescens, ATCC 274, was obtained and transferred directly into beef-extract broth without preliminary screening of the chromogenic variants. The static k_t value obtained with this culture was 0.034. Although this culture exhibited the same response under standard atmospheric conditions as that previously established, it was questionable whether such a culture would

be inherently stable because of the chromogenic and nonchromogenic variants present. The variants were separated from the culture being used routinely, designated as Culture A, and from the newly received culture, designated as Culture E. The criteria of separation was the color of the colonies: BR (blood-red), R (orange-red), P (pink), and W (white). The culture medium was nutrient agar. The distribution of variants in the undifferentiated cultures and the subsequent behavior of these variants when carried through a number of transfers in beef-extract broth are shown in Table XII.

In order to determine the static $\underline{k}_t$ values, the variant isolates were tested in the aerosol chamber under the standard atmospheric condition. These results are tabulated in Table XIII. The standard static $\underline{k}_t$ is found within the range of response shown by E-R and E-P. The variant E-R was continued as the standard test organism, and, from 11 subsequent determinations, the static $\underline{k}_t$ value of 0.031 was obtained. The variant E-P was not used further because it was found that the more highly pigmented variants possess the greater cultural stability. Table XII indicates the incipient differentiation of variants of E-P at the sixth transfer in beef-extract broth.

Additional characterization of the stable test culture was sought in the study of possible morphological and dimensional variation by the use of phase and electron microscopy.

Examination of Culture A, which had evidenced an abnormal response to the standard atmospheric condition, showed a wide variation in cell size, ranging from 1 to 2 microns in length and 0.7 to 1.0 micron in width to 15 microns in length and 1.0 to 1.5 microns in width. Many of the longer filamentous-type cells showed loci of increased density to the electron beam of the electron microscope.

TABLE XII

DISTRIBUTION OF COLOR VARIANTS OF
S. marcescens, ATCC 274, AFTER FOUR DAYS AT 20° C

Number of Broth Transfers	Culture	Number of Various Colony Forms				Total
		BR	R	P	W	
100	A	157	797	43	3	1,000
20	E	0	1,431	27	2	1,500
1	A-BR	1,700	0	0	0	1,700
1	A-R	0	2,300	0	0	2,300
1	A-P	0	0	2,000	0	2,000
1	A-W	0	0	-----	1,500	1,500
1	E-BR	1,927	27	229	17	2,000
1	E-R	0	1,200	0	0	1,200
1	E-P	0	0	1,984	16	2,000
1	E-W	0	0	4	1,996	2,000
6	A-R	0	4,489	0	11	5,500
6	A-W	0	16	0	4,184	4,200
6	E-BR	303	3,338	115	44	3,800
6	E-R	0	10,970	1	29	11,000
6	E-P	3	254	2,692	51	3,000
6	E-W	0	0	51	4,949	5,000
45	A-R	0	1,010	15	150	1,175
45	A-W	0	0	11	800	811
45	E-R	0	1,200	0	0	1,200
45	E-P	0	955	162	76	1,193
45	E-W	0	0	112	1,000	1,112

TABLE XIII

DIE-AWAY RATES, AIR-BORNE VARIANTS OF S. marcescens, ATCC 274, AT 68° F, 65 PER CENT RELATIVE HUMIDITY

Number of Broth Transfers	Calory Forms							
	Blood-Red		Red		Pink		White	
	A†	E	A	E	A††	E	A	E
3	-	0.042	0.005	0.017	-	0.035	0.005	0.039
6	-	0.053	0.054	0.036	-	0.037	0.008	0.047
7	-	0.049	0.010	0.021	-	0.023	0.005	0.054
8-19	-	-----	-----	0.031	-	-----	-----	-----

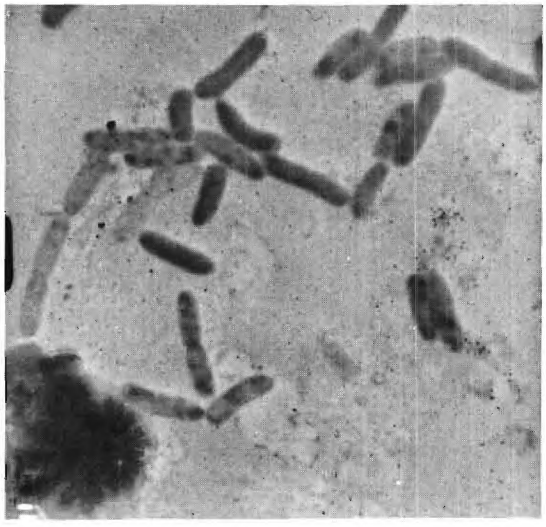
† Too few air-borne to obtain values.

†† After two isolations, cultures lost the pink color, became predominantly red and were discarded.

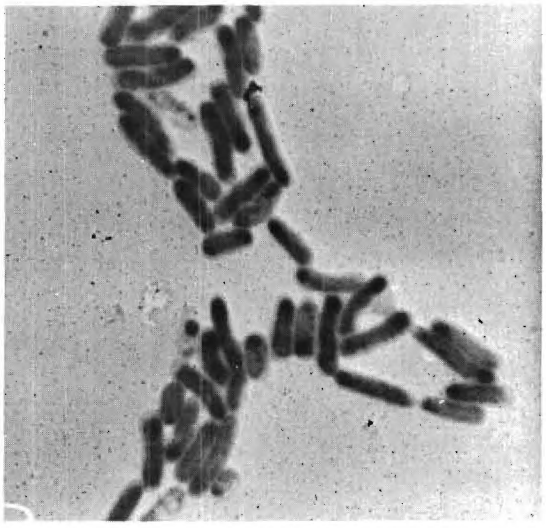
Culture E showed a more homogeneous population with cells having lengths ranging from 1.0 to 2.5 microns and widths ranging from 0.7 to 1.0 micron. Electron micrographs of this culture disclosed a uniform intracellular density.

The separation of the variants from Culture A and Culture E provided a more detailed examination of the cell types as correlated with colony pigmentation. Electron photomicrographs of third-isolate variants from nutrient agar surface colonies show a significant morphological and size variation among the variants of Culture A. These variants also appear quite different from the corresponding variants of Culture E which does not, however, exhibit a correspondingly wide range of cellular differentiation among it variants. The pictures of the variants of Culture A are shown in Figure 16; those of Culture E, in Figure 17.

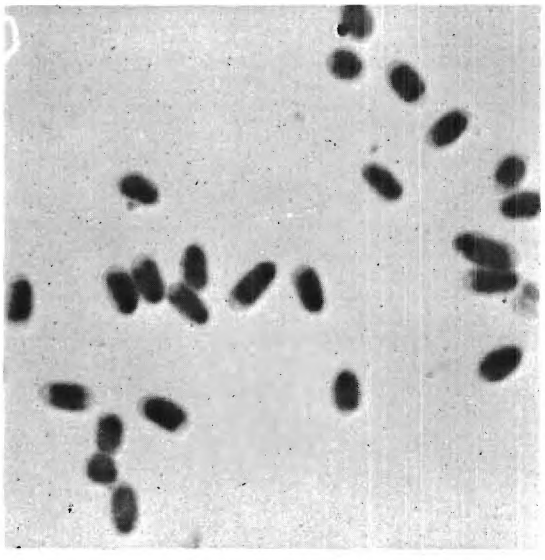
The correlated findings of morphology, cell dimension, and chromogenicity, together with the static k_t determinations, form the basis for monitoring the



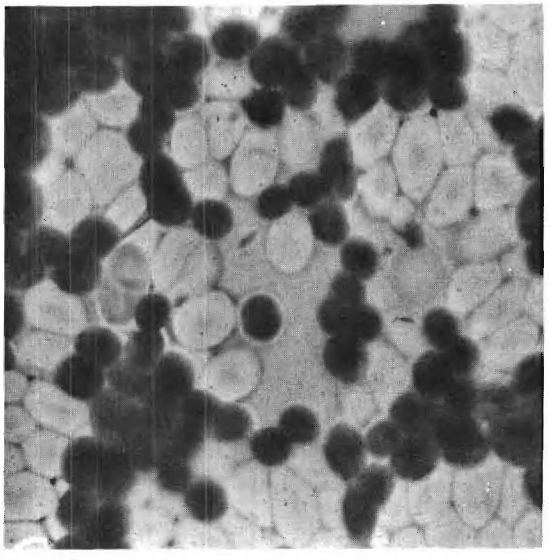
A-BR



A-R

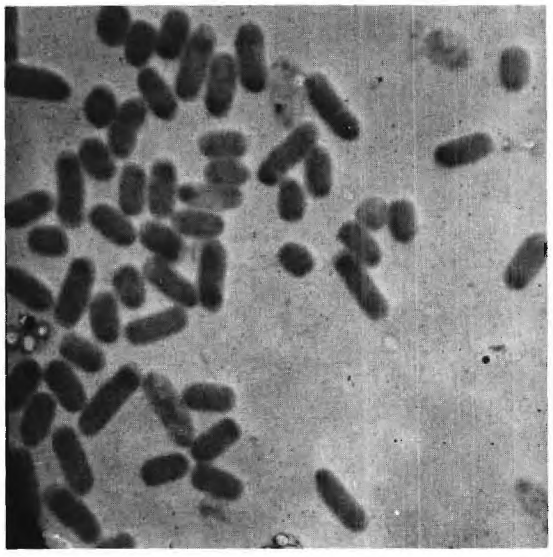


A-P

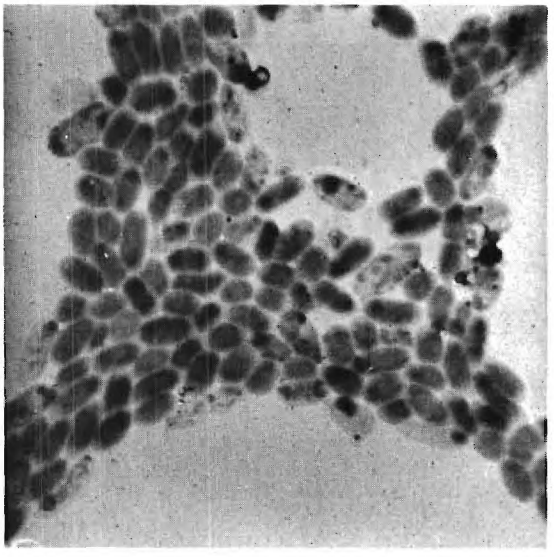


A-W

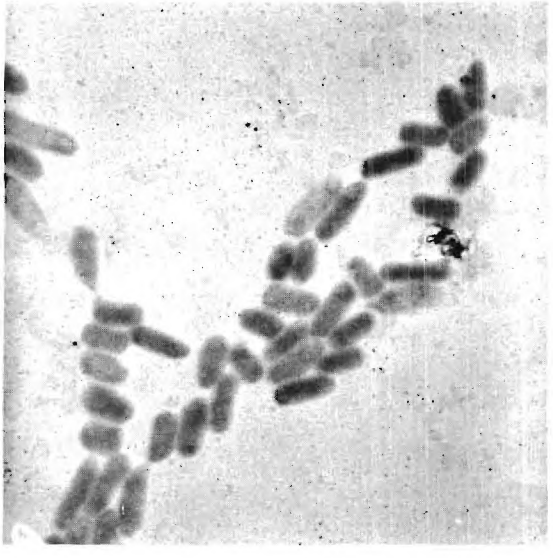
Figure 16. Electron Micrographs of the Variants of Culture A.



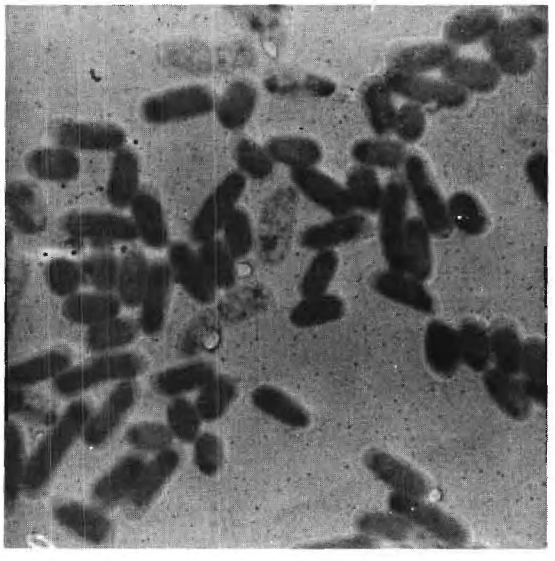
E-BR



E-R



E-P



E-W

Figure 17. Electron Micrographs of the Variants of Culture E.

test culture and enable a direct recovery of the test culture at such times when deterioration may be indicated. Storage of the variants on nutrient agar butts with mineral oil overlay at 5° C has proven satisfactory.

S. marcescens has been widely used as a test organism in the study of airborne microorganisms and has proven to be excellent for this purpose. However, as can be be deduced from the data presented, adequate monitoring of the particular variant being used is imperative if results are to be consistent. Although many other workers have used this organism, they have omitted the designation of the culture strain in reporting their results, and, therefore, it is impossible to determine if variations were encountered. Doubtless, other microorganisms used in similar studies have varying degrees of cultural stability and, therefore, exhibit differential responses when exposed to various atmospheres. The demonstrated differential responses among the isolated variants of S. marcescens, ATCC 274, serve to delineate the basic problems inherent in bacterial test organisms. They indicate that studies of the relative behavior of different genus and species should be extended to include the possible variants which may show as great a responsive difference as the different species exhibit. More detailed cultural studies may resolve some of the apparent disagreement in results reported by different workers who utilize, supposedly, the same organism.